APPENDIX A

PATENT
Customer No.: 32425

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
William WOLD *et al.*

Serial No.: 10/810,063

Filed: March 26. 2004

For: ADENOVIRUS REPLICATION-COMPETENT VECTORS EXPRESSING TRAIL

Group Art Unit: 1635

Examiner: Brian A. Whiteman

Atty. Dkt. No.: INGN:106US

Confirmation No.: 8527

CERTIFICATE OF ELECTRONIC TRANSMISSION
37 C.F.R. § 1.8

I hereby certify that this correspondence is being electronically filed with the United States Patent and Trademark Office via EFS-Web on the date below:

5/25/07
Date

Monica V. De La Paz

DECLARATION OF WILLIAM S. M. WOLD, UNDER 37 C.F.R. §1.132

I, William S. M. Wold, hereby declare as follows:

1. I am a Canadian citizen residing at 1609 Adgers Wharf Drive, Chesterfield, MO 63017.

2. I hold the position of Professor and Chair, Department of Molecular Microbiology and Immunology, Saint Louis University School of Medicine, St. Louis, MO. I am the first named inventor of the above-referenced patent application.

3. I have reviewed the specification of the above-referenced patent application ("the application") and the pending claims. I understand that the claims currently pending are directed generally to methods of treating a subject with a hyperproliferative cell disorder, such as cancer, by administering to the subject an adenovirus vector that is replication-competent in cancer cells, wherein the adenovirus vector includes a TRAIL coding region and an ADP coding region.

4. I understand that the examiner has rejected pending claims 23-38, 41, 43-47, and 49 as being obvious over Wold (U.S. Patent 6,627,190) in view of Walczak (Nature Med., 5(2):157-163, 1999). I am the first named inventor of Wold.

5. The invention set forth in the claims would not be obvious over Wold in view of Walczak. The disclosure in Wold makes no reference to TRAIL expression by any viral vector. While Walczak provides information regarding the ability of TRAIL to induce apoptosis of certain cell lines, it provides no particular suggestion or motivation to indicate that adenoviral vectors that express TRAIL and ADP can be successfully grown in cell lines or applied as an anticancer therapy. Walczak teaches that TRAIL has the ability to induce apoptosis in many cell lines, and that TRAIL has cytotoxic activity against a wide variety of transformed cell lines. *See, e.g.*, abstract of Walczak. Indeed, in view of the apoptosis-inducing ability of TRAIL, it is possible that expression of TRAIL may result in apoptosis of the host cells, which would be deleterious to virus replication if it should occur before replication is complete. *See* Chiou and White, Virology 244:108-118, 1998 (Exhibit A). For the same reason, expression of TRAIL may be deleterious to application of any such vector as an anticancer agent if it results in apoptosis of an infected cancer cell before virus replication is complete. This would defeat the purpose of Wold's goal of generating oncolytic vectors that can "replicate and spread throughout the tumor not just in the initially infected cells as in the case with replication-defective vectors." Wold, col. 2, lines 51-55.

6. A publication of Tollefson *et al.* (J. Virology, 75(19):8875-8887, 2001; Exhibit B) reports that TRAIL-induced apoptosis can be inhibited by certain adenoviral proteins, including E1B-19K. There is no specific discussion in the Wold disclosure that its adenoviral

vectors express E1B-19K. Thus, it is possible that expression of TRAIL in a vector of Wold could have resulted in apoptosis of the infected cell.

7. Further, Wold does not disclose whether insertion of another gene (complementary DNA) into the E3 region of the vector either 5' or 3' to the ADP gene will result in expression of the new gene or will preclude efficient expression of ADP. For example, since both ADP and TRAIL are expressed from the same adenovirus major late promoter via alternatively spliced mRNAs in some of the vectors of the present invention, it was possible that splicing into the ADP, TRAIL, or both coding sequences might not occur. Still further, the mechanism by which ADP functions to facilitate the efficient lysis (disruption) of infected cells at the culmination of the virus infection is unknown. Wold does not disclose whether expression of TRAIL may negate, augment, or modify the function of ADP. If TRAIL negates the function of ADP, then the cell lysis-promoting activity of ADP expression by the vector would be lost. If TRAIL augmented the function of ADP, then the infected cells may lyse before virus replication is complete. Therefore, unless and until the actual studies are conducted to prepare an adenoviral vector that includes a TRAIL coding region and an ADP coding region and evaluate its anti-cancer efficacy, one of ordinary skill in the art would not be able to predict with any degree of certainty whether such a vector can be successfully applied as an anticancer agent.

8. I understand that the Examiner has also rejected claims 23-38, 44-47, and 49 as being obvious over Henderson (U.S. Patent 6,197,293) taken with Griffith (U.S. Patent 6,900,185).

9. The invention set forth in the claims would not be obvious over Henderson in view of Griffith. Although Henderson teaches adenoviral vectors that are replication-competent,

it does not provide any teaching or suggestion regarding expression of TRAIL. Griffith, while teaching a method for inhibiting tumor cell growth in a mammal by administering TRAIL via a viral vector, does not appear to teach or suggest us of a replication-competent viral vector. Furthermore, as with the Wold patent and Walczak references discussed above, Griffith fails to provide any particular suggestion or motivation to indicate a replication-competent adenoviral vector such as taught by Henderson that expresses TRAIL and ADP can be successfully grown in cell lines or applied as an anticancer therapy. As discussed above, it was known in the field that TRAIL has the ability to induce apoptosis in many cell lines, and that TRAIL has cytotoxic activity against a wide variety of transformed cell lines. *See, e.g.*, abstract of Walczak. Indeed, in view of the apoptosis-inducing ability of TRAIL, it is possible that expression of TRAIL may result in apoptosis of the host cells, which would be deleterious to virus replication if it should occur before replication is complete. *See* above discussion regarding Chiou and White. Furthermore, even if TRAIL was expressed in one of the vectors taught by Henderson, it is possible that the infected cell could undergo apoptosis. As discussed above, Tollefson *et al.* (Exhibit B) teaches that TRAIL-induced apoptosis can be inhibited by certain adenoviral proteins, including E1B-19K. It is not clear whether the Henderson vectors express E1B-19K. Since the Henderson vectors use a prostate cancer-specific promoter to drive expression of the Ad E1A gene, and in some vectors they also use another prostate-specific promoter to drive expression of the E1B genes, it is possible that even if the vectors expressed E1B-19K, not enough E1B-19K would be expressed to inhibit TRAIL-induced apoptosis. Therefore, infection of a tumor cell with a vector of Henderson that expressed TRAIL could have resulted in apoptosis of

the infected cell, which could severely limit if not abolish the ability of the vector to replicate within infected cells.

10. For the same reasons discussed above in paragraph 7, until actual studies are conducted to prepare an adenoviral vector that includes a TRAIL coding region and an ADP coding region and evaluate its anti-cancer efficacy, one of ordinary skill in the art would not be able to predict with any degree of certainty whether such a vector can be successfully applied as an anticancer agent. The inventors made such a vector, and conducted studies to show that such vectors have anti-cancer efficacy.

11. Regarding the Examiner's argument that claims 23 and 40 are obvious in view of Henderson in view of Griffith and Bruder (Journal of Virology, 71:7623-7628, 1997), it is noted that an individual of skill in my field would not have found the invention set forth in these claims to be obvious. For the reasons discussed above regarding Henderson and Griffith, there would be no suggestion or motivation to provide for a method of treating a cancer using a replication-competent adenoviral vector that includes a TRAIL encoding region and an ADP coding region. Furthermore, any vector of Henderson that is engineered to express TRAIL could have resulted in apoptosis of an infected cell, which would be deleterious to adenoviral replication and application of the vector as an oncolytic vector. Bruder does not make the claimed invention obvious because like Griffith, it does not pertain to replication-competent vectors. Therefore, an individual with ordinary skill in my field would not have found the claimed invention obvious based on Henderson, Griffith, and Bruder.

12. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like

so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of this application or any patent issued thereon.

Date: May 23, 2007 William S. M. Wold

William S. M. Wold

EXHIBIT A

VIROLOGY 244, 108–118 (1998)
ARTICLE NO. VY989077

Inhibition of ICE-like Proteases Inhibits Apoptosis and Increases Virus Production during Adenovirus Infection

Shiun-Kwei Chiou and Eileen White*,[1]

*Center for Advanced Biotechnology and Medicine and *Department of Molecular Biology and Biochemistry and the Cancer Institute of New Jersey, Rutgers University, 679 Hoes Lane, Piscataway, New Jersey 08854*

Received November 11, 1997; returned to author for revision December 4, 1997; accepted February 10, 1998

Interleukin-1b converting enzyme (ICE)-related cysteine proteases are required for E1A-induced, p53-dependent apoptosis in baby rat kidney (BRK) cells. Adenovirus E1B 19K protein, which is a potent inhibitor of apoptosis, inhibits activation of these proteases in BRK cells. E1A expression induces apoptosis during infection of human cells by mutant adenoviruses which contain nonfunctional E1B 19K. The question arises as to whether ICE-related proteases are involved in E1A-induced apoptosis during mutant adenovirus infection of human cells. To test the involvement of the cysteine proteases in E1A-induced apoptosis during productive adenovirus infection of HeLa cells, we examined whether Z-VAD-FMK, an inhibitor of ICE-related proteases, can inhibit apoptosis induced by mutant adenovirus which lacks functional E1B 19K. Z-VAD-FMK inhibited E1A-induced apoptosis in adenovirus-infected Hela cells, suggesting that the ICE family proteases are involved in this apoptosis pathway. Z-VAD-FMK also inhibited cleavage of substrates such as cysteine protease CPP32 and nuclear lamins, whereas cleavage of poly(ADP-ribose) polymerase was partially inhibited during infection with an E1B 19K mutant. Inhibition of apoptosis by Z-VAD-FMK significantly enhanced production of infectious adenovirus and attenuated virus release. Thus apoptosis may be a method for the host cell to limit virus production and release at the end of the infection cycle.

INTRODUCTION

During adenovirus infection, E1A expression induces apoptosis, while E1B 19K expression inhibits apoptosis. Therefore, infection of HeLa cells by wild-type adenovirus, which expresses both E1A and E1B 19K, does not induce apoptosis (White *et al.*, 1991, 1992). Infection of HeLa cells with mutant adenovirus lacking functional E1B 19K produces massive apoptosis, and this cell death is dependent on E1A expression (Waga *et al.*, 1994; White *et al.*, 1984, 1991; White and Stillman, 1987). Cleavage of PARP, a known substrate for interleukin-1β converting enzyme (ICE)-like cysteine proteases, has been observed previously in E1B 19K mutant adenovirus-induced cell death in KB cells (Boulakia *et al.*, 1996) and in Sindbis virus-induced apoptosis of neuroblastoma cells (Ubol *et al.*, 1996), implicating the involvement of cysteine proteases in viral infection-induced apoptosis. Furthermore, inhibition of cysteine protease activity has been shown to enhance HIV replication by inhibiting apoptosis (Chinnaiyan *et al.*, 1997). Thus cysteine protease activity appears to be an important factor in the regulation of apoptosis and the progression of the virus life cycle.

The study of the genetic control of apoptosis in the nematode *Caenorhabditis elegans* (*C. elegans*) first suggested the involvement of cysteine proteases in apoptosis. Specifically, the *C. elegans* death protein Ced-3, which is one of the required factors for execution of the apoptosis program in nematodes (Ellis and Horvitz, 1986; Hengartner and Horvitz, 1994), is a cysteine protease (Xue *et al.*, 1996) homologous to the mammalian ICE family of cysteine proteases. This suggests a role for these ICE-related proteases in mammalian apoptosis (Yuan *et al.*, 1993). Numerous mammalian proteases related to ICE have been identified. These include CPP32/Yama/apopain (Fernandes-Alnemri *et al.*, 1994; Nicholson *et al.*, 1995; Tewari *et al.*, 1995), Mch3/CMH1 (Fernandes-Alnemri *et al.*, 1995b; Lippke *et al.*, 1995), Mch2 (Fernandes-Alnemri *et al.*, 1995a), Mch4 (Fernandes-Alnemri *et al.*, 1996), Mch5 (Fernandes-Alnemri *et al.*, 1996), ICE rel II/Tx/ICH-2 (Faucheu *et al.*, 1995; Kamens *et al.*, 1995; Munday *et al.*, 1995), ICE rel III (Munday *et al.*, 1995), and Ich-1 (Wang *et al.*, 1994). These proteases are now collectively referred to as "caspases" (Alnemri *et al.*, 1996). An insect caspase, *Spodoptera frugiperda* caspase-1, has also been found recently, which is inhibitable by the anti-apoptotic baculovirus p35 protein (Ahmad *et al.*, 1997). *Drosophila melanogaster* also encodes caspases that are required for apoptosis, such as drICE and DCP-1 (Fraser and Evan, 1997;

[1] To whom correspondence and reprint requests should be addressed at Center for Advanced Biotechnology and Medicine, 679 Hoes Lane, Piscataway, New Jersey 08854. Fax: (732) 235-5795. E-mail: ewhite@mbcl.rutgers.edu.

0042-6822/98 $25.00

Harvey *et al.*, 1997; Song *et al.*, 1997). Thus caspases appear to be important for apoptosis across different phyla.

Presumably, activation of caspases during apoptosis results in cleavage of a number of cellular substrates which lead to the apoptotic phenotype. Substrates for the caspases include the retinoblastoma tumor suppressor protein (pRb), interleukin-1β, nuclear lamins, and poly-(ADP-ribosepolymerase (PARP) (Enari *et al.*, 1996; Janicke *et al.*, 1996; Lazebnik *et al.*, 1994; Orth *et al.*, 1996). Although the roles of interleukin-1β and PARP cleavage in apoptosis are unclear, lamin cleavage has been shown to be an important event in the apoptotic process, since overexpressed uncleavable lamins delay DNA degradation and cell death (Rao *et al.*, 1997). Specific caspases which cleave pRb have not been identified, although pRb degradation has been observed during apoptosis induced by TNF-α, Fas, and staurosporine, and inhibitors of caspases have been shown to block both pRb cleavage and apoptosis (Janicke *et al.*, 1996; Tan *et al.*, 1997). Since pRb has known anti-apoptosis activity (Berry *et al.*, 1996; Haas-Kogan *et al.*, 1995; Howes *et al.*, 1994; McConkey *et al.*, 1996), degradation of pRb may also be an important event in apoptosis.

Overexpression of caspases has been shown to induce apoptosis and cellular as well as viral inhibitors of apoptosis inhibit the activation or activity of caspases (Boulakia *et al.*, 1996; Bump *et al.*, 1995; Komiyama *et al.*, 1994; Kumar, 1995; Martin and Green, 1995; Nicholson, 1996; Sabbatini *et al.*, 1997; Tewari and Dixit, 1995; Xue and Horvitz, 1995). Cleavage of substrates by specific caspases is blocked by viral inhibitors such as CrmA from cowpox virus and p35 from baculovirus (Bump *et al.*, 1995; Komiyama *et al.*, 1994; Tewari and Dixit, 1995; Xue and Horvitz, 1995). The Bcl-2 cellular oncoprotein and the adenovirus E1B 19K both inhibit activation of caspases (Boulakia *et al.*, 1996; Sabbatini *et al.*, 1997).

Synthetic peptide inhibitors have been developed and are designed based on conserved amino acid sequences in the substrate cleavage sites of the different proteases. Substrate cleavage sites for the caspases generally contain a tetrapeptide motif. ICE cleaves most effectively at the tetrapeptide sequence Tyr-Val-Ala-Asp (YVAD), whereas CPP32 most effectively cleaves at Asp-Glu-Val-Asp (DEVD) (Zhu *et al.*, 1995). Synthetic peptide aldehyde inhibitors containing these sequences have been useful in identifying the role of specific caspases in apoptosis. An aspartate residue in the tetrapeptide sequence at the P1 position and at least four amino acids on the amino-terminal side of the cleavage site are required for cleavage to occur. Removal of the amino acid in the P4 position in the peptide aldehyde inhibitor benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone (Z-VAD-FMK) renders it a general inhibitor of caspases (Zhu *et al.*, 1995).

It has been shown earlier that Z-VAD-FMK inhibits E1A-induced apoptosis in BRK cells, suggesting that caspases are involved in this apoptosis pathway (Sabbatini *et al.*, 1997). Nuclear lamin cleavage, which is indicative of Mch2 activity, and active CPP32 subunits have been observed during E1A-induced apoptosis in baby rat kidney (BRK) cells (Rao *et al.*, 1996; Sabbatini *et al.*, 1997). Thus Mch2 and CPP32 are involved in this apoptosis pathway. Furthermore, E1B 19K expression blocks E1A-induced apoptosis and also inhibits activation of these caspases in BRK cells (Rao *et al.*, 1996; Sabbatini *et al.*, 1997).

E1B 19K is an anti-apoptosis member of the Bcl-2 family of apoptosis regulators, which function upstream of caspase activation (Boulakia *et al.*, 1996; Rao *et al.*, 1996). The mechanism of inhibition of caspases by anti-apoptotic Bcl-2 family members is unclear. It is possible that anti-apoptotic Bcl-2 family members express a survival signal that inhibits caspase activation or that they inhibit a death signal mediated by pro-apoptotic Bcl-2 family members Bax, Nbk/Bik, and Bak to activate caspases (reviewed in Rao and White, 1997).

Since caspases play a role in E1A-induced apoptosis in BRK cells, the question arises as to whether E1A-induced apoptosis in mutant adenovirus-infected HeLa cells also involves caspases. To test the involvement of the caspases in E1A-induced apoptosis during adenovirus infection of HeLa cells, we evaluated whether the Z-VAD-FMK peptide inhibitor could inhibit apoptosis during E1B 19K mutant adenovirus infection of HeLa cells. Z-VAD-FMK inhibited E1A-induced apoptosis as well as cleavage of specific substrates such as CPP32 and nuclear lamins. PARP cleavage was reduced but not inhibited upon Z-VAD-FMK treatment. The consequence of inhibition of apoptosis by Z-VAD-FMK was to increase infectious virus production and to attenuate virus release from the infected cells.

In both wild-type and 19K mutant adenovirus infection, the efficiency of virus release from the host HeLa cells was similarly decreased by treatment with Z-VAD-FMK. Thus inhibition of apoptosis by Z-VAD-FMK diminished virus release regardless of whether E1B 19K is present. This suggested that inhibition of apoptosis is required but E1B 19K activity is dispensable for blocking virus release.

RESULTS

Inhibition of caspase activity inhibits E1A-Induced apoptosis during adenovirus infection of HeLa cells

Of the caspases, ICE, CPP32, and Mch2 activity has been detected in cells undergoing apoptosis (reviewed in Fraser and Evan, 1996). Stable expression of CrmA did not inhibit E1A-induced apoptosis in infected HeLa cells (data not shown), suggesting that ICE itself may be dispensable for this form of apoptosis or that E1A triggers redundant pathways in HeLa cells. To determine whether ICE-like

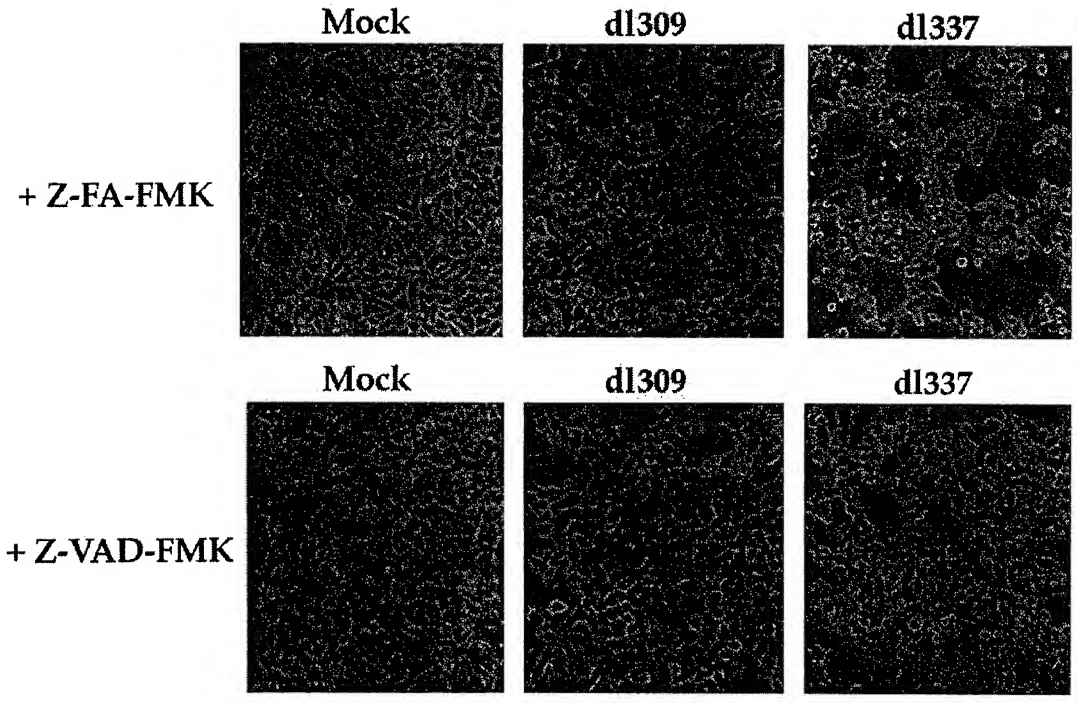

FIG. 1. Z-VAD-FMK treatment inhibits appearance of the *cyt* phenotype indicative of apoptosis in adenovirus-infected HeLa cells. HeLa cells were mock infected or infected with the Ad5*dl*309 or Ad5*dl*337 viruses at a m.o.i. of 100 PFU/cell. 25 mm of Z-VAD-FMK or Z-FA-FMK was added to each infection at 6 h postinfection. At 48 h postinfection the appearance of the *cyt* phenotype was monitored. The peptide treatments are indicated on the left. Virus infections are indicated above each panel. Original magnification, ×25.

caspases in general are involved in E1A-induced apoptosis during adenovirus infection, we tested whether inhibition of caspase activity by Z-VAD-FMK could inhibit induction of apoptosis during infection of HeLa cells by mutant adenovirus lacking functional E1B 19K.

HeLa cells were mock infected, infected with the wild-type Ad5*dl*309 virus, or infected with the Ad5*dl*337 virus, which contains a deletion in the E1B 19K coding sequence and induces apoptosis (Pilder *et al.*, 1984; White *et al.*, 1984; White and Stillman, 1987). Infected HeLa cells were then treated in parallel with either the Z-FA-FMK control peptide or the Z-VAD-FMK peptide inhibitor. Apoptosis induction was monitored by observing the appearance of extreme changes in the cell morphology (*cyt* phenotype) and DNA degradation (*deg* phenotype).

Mock-infected cells treated with either control peptide or Z-VAD-FMK did not exhibit any *cyt* phenotype (Fig. 1). HeLa cells infected by Ad5*dl*309 and treated with either Z-FA-FMK or Z-VAD-FMK peptide also did not exhibit the *cyt* phenotype (Fig. 1). Thus peptide treatment did not produce nonspecific morphological effects on mock- or wild-type virus-infected HeLa cells. Cells that were infected by Ad5*dl*337 and were treated with the control peptide exhibited the *cyt* phenotype, apparent from the extensive rounding and detaching of cells from the dish (Fig. 1). This was expected since the control peptide would not protect the cells from induction of apoptosis by Ad5*dl*337 virus infection. In contrast, treatment of Ad5*dl*337-infected cells with the Z-VAD-FMK inhibitor blocked induction of the pronounced morphological changes characterized by the *cyt* phenotype (Fig. 1). Thus inhibition of caspases inhibited morphological indications of apoptosis during mutant adenovirus infection, suggesting that caspases play a role in those events caused by E1A-induced apoptosis during adenovirus infection of human cells.

Analysis of the *deg* phenotype showed similar results. Mock-infected cells and cells infected by Ad5*dl*309 did not display DNA fragmentation, and treatment with either peptide had no effect (Fig. 2, left panel). The *deg* phenotype was observed in cells infected by Ad5*dl*337 treated with the control peptide, as indicated by the appearance of DNA fragmentation, but was not observed in cells treated with the Z-VAD-FMK inhibitor (Fig. 2, left panel). Restriction analysis of viral DNA from infected cells indicated efficient viral replication had taken place to a similar extent in all the infections (Fig. 2, right panel), indicating that Z-VAD-FMK did not block apoptosis by inhibiting virus replication. Thus, by inhibiting ICE-like caspases, two distinct apoptosis phenotypes (*cyt* and *deg*) in mutant adenovirus-infected HeLa cells were inhibited.

The Z-VAD-FMK inhibitor blocks the proteolysis of specific substrates during apoptosis in adenovirus-infected HeLa cells

Inhibition of apoptosis by Z-VAD-FMK during E1B 19K mutant adenovirus infection implies that the protection

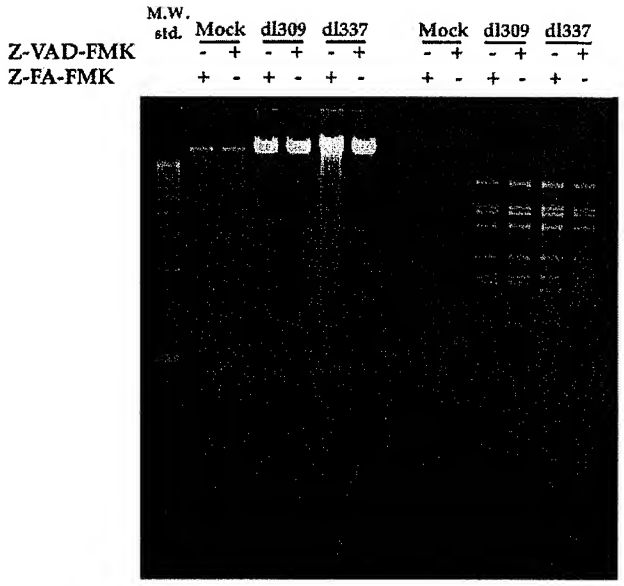

FIG. 2. Z-VAD-FMK treatment inhibits DNA degradation (*deg* phenotype) during apoptosis in adenovirus-infected HeLa cells. HeLa cells were mock infected or infected and then treated with peptides as described in Fig. 1. Concurrent to the observation of the *cyt* phenotype in Fig. 1, Hirt DNA was isolated from each infection and peptide treatment. The *deg* phenotype was monitored by agarose gel electrophoresis. Treatment with control peptide or Z-VAD-FMK is indicated on the left. Viral infections are indicated above each lane. Panels: left, undigested DNA; right, *Hin*dIII-digested DNA.

from cell death is due to blockage of caspase activity in the infected cells. Proteolytic activation of CPP32 and cleavage of nuclear lamins and PARP have been shown to occur during E1A-induced, p53-mediated apoptosis in BRK cells (Rao *et al.*, 1996; Sabbatini *et al.*, 1997), and Z-VAD-FMK inhibits these cleavage events (Sabbatini *et al.*, 1997). To determine whether these proteolytic events also occur in E1B 19K mutant adenovirus-infected HeLa cells undergoing apoptosis, and whether Z-VAD-FMK treatment can inhibit cleavage of these substrates, CPP32, nuclear lamins, and PARP were monitored concurrently with manifestation or inhibition of the *cyt* and *deg* phenotypes.

Activation of CPP32 entails processing of the inactive zymogen into p17 and p11 active subunits (Nicholson *et al.*, 1995). Detection of either subunit on a Western blot indicates that CPP32 is processed. Extracts from apoptosing BRK cells and live BRK cells were used as positive and negative controls, respectively, for the presence of the p17 active subunit of CPP32 (Fig. 3). The p17 subunit was absent in cell extracts from mock-infected and Ad5*dl*309-infected cells treated with either Z-FA-FMK or Z-VAD-FMK (Fig. 3). Thus CPP32 was inactive in mock-infected and adenovirus-infected HeLa cells inhibited from undergoing apoptosis by E1B 19K expression. Similar findings were reported indicating that E1B 19K acts upstream to inhibit caspase activation in BRK cells (Sabbatini *et al.*, 1997). In cells infected by Ad5*dl*337 in the presence of control peptide that were undergoing apoptosis, p17 was present in abundance (Fig. 3), whereas treatment of Ad5*dl*337-infected cells with the Z-VAD-FMK inhibitor blocked the appearance of p17 (Fig. 3). Thus CPP32 was activated in cells undergoing apoptosis and inhibited from activation when cells were rescued from apoptosis by Z-VAD-FMK.

Cleavage of nuclear lamins A/C into 47K and 37K proteolysis products has been observed in apoptosing cells (Ankarcrona *et al.*, 1996; Oberhammer *et al.*, 1994; Orth *et al.*, 1996; Rao *et al.*, 1996; Takahashi *et al.*, 1996). In mock-infected and Ad5*dl*309-infected HeLa cells treated with either the control or the Z-VAD-FMK peptide, lamin cleavage was undetectable by Western blot analysis (Fig. 3), as was expected since these cells were viable. A band migrating close to 47K is observed in the lane representing mock infection treated with Z-VAD-FMK (Fig. 3). However, this band migrates slightly below 47K, suggesting that it is only a background band. Also, the 37K lamin cleavage product is absent in the lane, suggesting that the enzymatic activity which produces this product is absent. The cleavage products of lamins

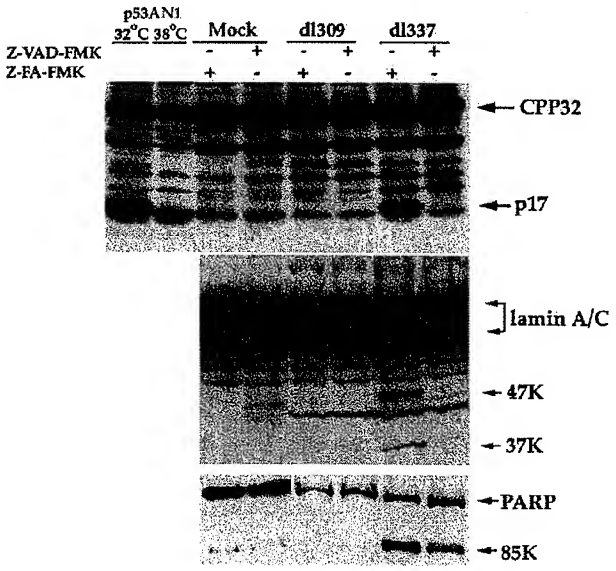

FIG. 3. Inhibition of cleavage of CPP32, nuclear lamins, and PARP during apoptosis in mutant adenovirus-infected HeLa cells by Z-VAD-FMK treatment. Whole cell lysates were made from the mock- or virus-infected HeLa cells treated with either Z-VAD-FMK or Z-FA-FMK (Fig. 1), and cleavage products of CPP32, laminA/C, and PARP were monitored by Western blot analysis. The CPP32-p17 polyclonal antibody was used to detect the p17 active subunit of CPP32. The monoclonal antibodies C2-10 and lE4 were used to detect PARP and lamin A/C, respectively. p53AN1, a BRK cell line transformed by E1A and a temperature-sensitive p53(val135) mutant. 32°C, protein extract from apoptosing p53AN1 cells serving as positive control for CPP32 activation. 38°C, protein extract from healthy p53AN1 cells serving as negative control for CPP32 activation. Viral infections are indicated at the top of the panel; (+) presence of the indicated peptide and (−) absence of the indicated peptide. The full-length CPP32 and the active p17 subunit are indicated on the right. The full-length and cleavage products of both PARP and lamin A/C are also indicated on the right.

A/C were detected in Ad5*dl*337-infected cells treated with control peptide and undergoing apoptosis, but not in Ad5*dl*337-infected cells rescued from apoptosis by the Z-VAD-FMK peptide inhibitor (Fig. 3).

Cleavage of PARP into an 85K product has also been observed during apoptosis, as a function of CPP32, Mch2, Mch3, or Ich-1 activity (Fernandes-Alnemri *et al.*, 1994, 1995a,b; Nicholson *et al.*, 1995; Tewari *et al.*, 1995; Wang *et al.*, 1994). In mock-infected cells, the 85K PARP cleavage product was observed at very low background levels (Fig. 3). Since a small amount of background PARP cleavage was observed in both Z-FA-FMK- and Z-VAD-FMK-treated cells, it appeared to be a nonspecific event. What caused the background PARP cleavage is unclear. It could be that there is basal PARP cleavage activity present in the live cell or that there are background levels of cell death. If the PARP cleavage is due to background cell death, it is unlikely to be caused by toxicity of the Z-VAD-FMK inhibitor alone since the level of PARP cleavage in both the control peptide- and the Z-VAD-FMK inhibitor-treated samples were equivalent. In Ad5*dl*309-infected HeLa cells, PARP cleavage was undetectable (Fig. 3), as expected since E1B 19K is present in a wild-type adenovirus infection, and is reported to function upstream of protease activation (Boulakia *et al.*, 1996; Rao *et al.*, 1996; Sabbatini *et al.*, 1997). In cells infected with Ad5*dl*337 that were undergoing apoptosis in the presence of control peptide, the 85K PARP cleavage product was detected in abundance, as expected since CPP32 was activated (Fig. 3), and lamin cleavage indicative of Mch2 activity was observed (Fig. 3). However, the 85K PARP cleavage product was also present in relative abundance in Ad5*dl*337-infected cells treated with Z-VAD-FMK, even though CPP32 activation and lamin cleavage were inhibited and cells were rescued from apoptosis (Fig. 3). It is conceivable that PARP cleaving proteases other than CPP32 were present. Alternatively, PARP cleavage may not be involved in apoptosis, since basal levels of the PARP cleavage product were detected in mock-infected cells, which were not undergoing apoptosis (Fig. 3).

Inhibition of apoptosis by Z-VAD-FMK increases virus yield

It has been suggested that apoptosis is a mechanism by which the host cell prematurely terminates viral infection. If this is true, than inhibition of apoptosis would allow infection to go to completion, thus increasing the total virus production. To determine whether inhibition of apoptosis by Z-VAD-FMK could increase virus production, the infectious virus yield from E1B 19K mutant adenovirus-infected and apoptosing cells were compared to that from infected cells rescued from apoptosis by Z-VAD-FMK. The infectious virus yield from wild-type adenovirus infection served as a control.

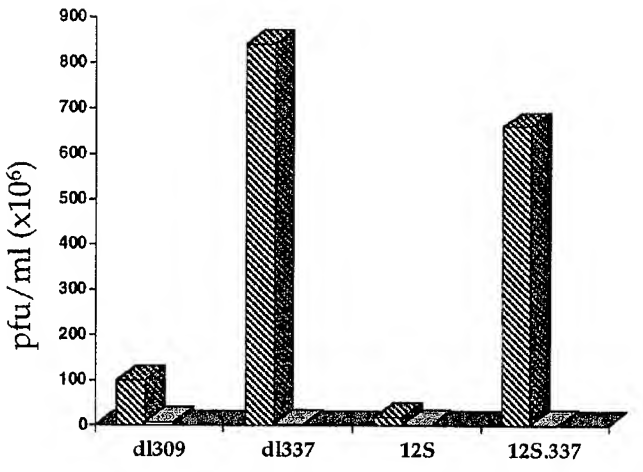

FIG. 4. Inhibition of apoptosis by Z-VAD-FMK causes an increase in virus production in HeLa cells. HeLa cells were infected with Ad5*dl*309, Ad5*dl*337, 12S, or 12S.337 viruses and then treated with either Z-FA-FMK or Z-VAD-FMK. When infection is complete as indicated by cells rounding up and floating off the surface of the dish, cells and culture medium were harvested and infectious virus yield was determined by plaque formation on 293 cells. Infection was complete after 48 h for Z-FA-FMK-treated Ad5*dl*309 and Ad5*dl*337 infections and after 72 h for Z-VAD-FMK-treated Ad5*dl*309 and Ad5*dl*337 infections and all the 12S and 12S.337 infections. The *Y* axis represents the virus yield in PFU($\times 10^3$)/ml. The *X* axis represents the different viral infections and peptide treatments. The gray columns represent infectious virus yields from infections treated with the Z-FA-FMK control peptide. Hatched columns represent infectious virus yield from infections treated with Z-VAD-FMK. Viruses used for each infection are indicated at the bottom of the graph.

The virus yield from Ad5*dl*309-infected cells treated with Z-VAD-FMK was 1.0×10^8 PFU/ml, which was 11 times greater than that from the same infection treated with control peptide (8.8×10^6 PFU/ml) (Fig. 4). Thus Z-VAD-FMK augmented virus replication independent of inhibition of apoptosis by E1B 19K.

The Ad5*dl*337-infected cells treated with Z-VAD-FMK produced a virus yield of 8.4×10^8 PFU/ml, which was about 300-fold greater than the virus yield from control peptide treatment (3.0×10^6 PFU/ml) (Fig. 4). Thus inhibition of apoptosis contributed even more greatly to an increase in virus yield in the absence of E1B 19K expression. It was unexpected that virus production by Ad5*dl*337 in the presence of ZVAD-FMK would be dramatically greater than with the wild-type adenovirus (Fig. 4). This suggested that the E1B 19K protein may suppress virus replication independent of its role as an apoptosis inhibitor.

A second set of replication-defective adenoviruses (12S and 12S.337) expressing the 12S E1A product were also evaluated in the same assay. Both 12S and 12S.337 viruses express only the 12S gene product of E1A, which lacks a region that transactivates expression of downstream viral genes and renders the virus partially defective for replication (Berk, 1986; Lillie *et al.*, 1987; Moran *et al.*, 1986; Moran and Mathews, 1987), but 12S.337 has a

deletion of the E1B 19K gene (White *et al.*, 1988). As a result, 12S.337 potently induces apoptosis in infected HeLa cells (White *et al.*, 1988; White and Stillman, 1987). The *cyt* and *deg* phenotypes are more pronounced in 12S.337-infected cells than in Ad5*dl*337-infected cells (White *et al.*, 1988; White and Stillman, 1987).

Z-VAD-FMK treatment of 12S-infected cells resulted in a virus yield of 1.76×10^7 PFU/ml, which was 10 times greater than that of the control peptide treatment (1.8×10^6 PFU/ml) (Fig. 4). Z-VAD-FMK treatment of 12S.337-infected cells produced a virus yield of 6.6×10^8 PFU/ml, which was about 200 times greater than that from the control peptide treatment (3.4×10^6 PFU/ml) (Fig. 4). As in the case with Ad5*dl*337, Z-VAD-FMK treatment greatly enhanced 12S.337 virus replication over and above that of the 12S virus. From both sets of infections, inhibition of apoptosis by Z-VAD-FMK significantly increased infectious virus yields, suggesting that inhibition of caspases and apoptosis contributes to completion of the adenovirus infection cycle and maximization of viral progeny production in the host HeLa cells.

Inhibition of apoptosis by Z-VAD-FMK attenuates release of viral particles into the environment

Another event in apoptosis in infected cells is premature cell lysis and release of virus into the extracellular environment. To test whether inhibition of apoptosis by Z-VAD-FMK has any effect on virus release from infected HeLa cells, the percentages of virus release from the Ad5*dl*309, 12S, Ad5*dl*337, and 12S.337 infections treated with Z-VAD-FMK were compared to those from the same infections treated with control peptide. Virus release from cells infected by Ad5*dl*309 and treated with Z-VAD-FMK was fourfold less than that from Ad5*dl*309-infected cells treated with control peptide (Fig. 5). With Z-VAD-FMK treatment, a fourfold reduction in virus release was observed in the Ad5*dl*337 infection (Fig. 5). The reduction in the percentage of virus release from Ad5*dl*309 infection was similar to that from Ad5*dl*337 infection. Thus the effect of Z-VAD-FMK on virus release was independent of E1B 19K, since functional E1B 19K is not expressed in Ad5*dl*337 infection. Compared to the control peptide treatment, Z-VAD-FMK treatment of 12S-infected cells resulted in a twofold reduction in virus release (Fig. 5). Compared to control peptide treatment, Z-VAD-FMK treatment of 12S.337-infected cells resulted in sixfold less virus release (Fig. 5). Thus, the reduction in the percentage of virus release by Z-VAD-FMK treatment was threefold greater in the 12S.337 infection than in the 12S infection. Surprisingly, there was a reduction in virus release in all the infections by Z-VAD-FMK treatment.

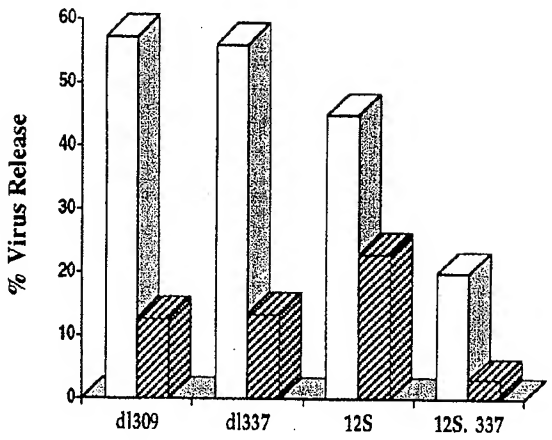

FIG. 5. Inhibition of apoptosis attenuates release of viral particles from infected HeLa cells. HeLa cells were infected with Ad5*dl*309, Ad5*dl*337, 12S, or 12S.337 viruses and treated with either Z-FA-FMK or Z-VAD-FMK. When infection is complete (see Fig. 4), cells were separated from the culture supernatant, the infectious virus yield in the supernatant and cell pellet were determined, and the percentage of virus release was calculated. The percentage of virus release was calculated as the (virus yield in the supernatant/total virus yield) ×100. The *Y* axis represents the percentage of virus yield. The *X* axis represents the different viral infections and peptide treatments. Open columns represent the percentage of virus release from infected cells treated with control peptide. Hatched columns represent the percentage of virus yield from infected cells treated with Z-VAD-FMK.

DISCUSSION

Inhibition of caspase activity in BRK cells blocks E1A-induced, p53-mediated apoptosis (Rao *et al.*, 1996; Sabbatini *et al.*, 1997). We report here that inhibition of caspase activity also blocks E1A-induced apoptosis during adenovirus infection of HeLa cells. Specifically, our results showed that CPP32 and caspases which cleave lamin and/or PARP and possibly Mch2, Mch3, and Ich-1 (Fernandes-Alnemri *et al.*, 1994, 1995a,b; Nicholson *et al.*, 1995; Orth *et al.*, 1996; Tewari *et al.*, 1995; Wang *et al.*, 1994) are likely involved in E1A-induced apoptosis. Our results also suggest that Z-VAD-FMK inhibits the apoptosis pathway at, or upstream of, CPP32 activation, since CPP32 cleavage was detected in apoptosing cells and absent in protected cells. If Z-VAD-FMK inhibits the pathway upstream of CPP32, this would suggest that caspase activation in infected HeLa cells occurs in a cascade fashion and that CPP32 activation depends on the activity of an upstream caspase. Z-VAD-FMK also blocks the caspase pathway upstream of the lamin cleavage activity, since lamin cleavage is detected in dying cells but not in protected cells.

E1A expression during adenovirus infection of HeLa cells has been shown to induce both p53-dependent and -independent forms of apoptosis (Chiou and White, 1997; Teodoro *et al.*, 1995). Also, the viruses used in this study contain intact E1B 55K. The observation that E1B 55K can inhibit p53-induced cell death (Marcellus *et al.*, 1996), but is insufficient to inhibit E1A-induced apoptosis in HeLa cells, suggests that a p53-independent apoptosis pathway is active during infection. Whether E1B 55K is sufficient to inhibit p53-induced apoptosis in our experiments

is unclear since both p53-dependent and -independent apoptosis are activated. Complete blockage of apoptosis in Ad5*dl*337-infected HeLa cells by Z-VAD-FMK suggested that caspases are essential for both p53-dependent and -independent forms of apoptosis in infected HeLa cells. There is evidence, however, that inhibition of caspase activity with the Z-VAD-FMK inhibitor does not inhibit apoptosis induced by DNA damage, which has been shown to require p53, in Rat-1 fibroblasts (Kastan *et al.*, 1991; McCarthy *et al.*, 1997). One explanation for this aparent inconsistency is that different cell types have been used to study inhibition of p53-dependent apoptosis by Z-VAD-FMK (McCarthy *et al.*, 1997; Sabbatini *et al.*, 1997), and different caspases may be expressed in the different cell types. There may be caspases in the p53-dependent apoptosis pathway in Rat-1 fibroblasts which are uninhibitable by Z-VAD-FMK. Another explanation is that the concentrations of Z-VAD-FMK used may be insufficient to completely inhibit apoptosis. A third explanation is that there are caspase-independent apoptosis pathways in Rat-1 fibroblasts.

Apoptosis induction is thought to be a host cell defense against viral infection. Inhibition of apoptosis has been reported to enhance production of human immunodeficiency virus (Antoni *et al.*, 1995; Chinnaiyan *et al.*, 1997; Sandstrom *et al.*, 1996), simian immunodeficiency virus (Chang *et al.*, 1993), and the baculovirus *Autographa californica* nuclear polyhedrosis virus (Clem and Miller, 1993) during infection of their respective host cells. Furthermore, adenovirus, Epstein–Barr virus, human herpes virus 8, herpes simplex virus, African swine fever virus, and baculovirus all encode specific genes which inhibit apoptosis (Afonso *et al.*, 1996; Brun *et al.*, 1996; Cheng *et al.*, 1997; Leopardi and Roizman, 1996; White *et al.*, 1992), reviewed in (Gooding, 1992; White, 1993; White and Gooding, 1994).

Loss of E1B 19K, which allows apoptosis to occur during adenovirus infection, is expected to result in less virus production. However, this is true for some, but not all, cell types (Pilder *et al.*, 1984). In HeLa and WI38 cells, loss of E1B 19K function in mutant adenoviruses promotes viral production rather than attenuates it (White *et al.*, 1988). In these cell types, E1B 19K suppresses E1A expression and dampens E1A-dependent viral gene expression (White *et al.*, 1988). E1A expression stimulates cell cycle progression, which is believed to provide the machinery for efficient virus replication (reviewed in Moran, 1993). Thus downregulation of E1A expression by E1B 19K may suppress cell cycle progression and virus replication (White *et al.*, 1988). The net result of E1B 19K expression during infection is that virus production is enhanced by inhibition of apoptosis but slowed by inhibition of cell cycle progression due to downregulation of E1A expression.

Inhibition of apoptosis by Z-VAD-FMK during E1B 19K mutant adenovirus infection of HeLa cells caused an increase in virus production. This observation is expected since Z-VAD-FMK inhibited apoptosis in the absence of any negative effects on virus replication due to the absence of E1B 19K. Z-VAD-FMK also slightly increased production of the wild-type Ad5*dl*309 and 12S viruses, which did not detectably induce premature cell death. This may be possible if E1B 19K expression alone does not completely inhibit apoptosis, but Z-VAD-FMK treatment gives additive protection from apoptosis. Another possibility is that E1B 19K inhibits apoptosis early during infection, but loses its protective function late during infection, thus allowing apoptosis to be reactivated. In this case, Z-VAD-FMK may inhibit the apoptosis late during infection, thus extending the adenovirus lifecycle to allow for more virus production. Alternatively, inhibition of caspases may enhance virus production independently of apoptosis inhibition.

Inhibition of apoptosis by Z-VAD-FMK also caused a reduction in the release of all the virus we tested from the host cell. This suggests that not only did premature virus released from the Ad5*dl*337 and 12S.337 infections occur by apoptosis, but also that the final release of viral particles from the Ad5*dl*309 and 12S infections may also have occurred by apoptosis. Alternatively, it is possible that caspases that are unrelated to apoptosis but inhibitable by Z-VAD-FMK are involved in the final virus release mechanism. A third possibility exists that the reduction in virus release is the result of a combination of increased virus production due to Z-VAD-FMK treatment and an unaltered rate of virus release by the host cell. In this case, neither apoptosis nor caspases need be required for virus release.

The possibility that apoptosis is responsible for release of virus particles at the end of a wild-type virus infection supports the idea that E1B 19K is not functional at late stages during infection. The primary function of E1B 19K may be inhibition of E1A-induced apoptosis early in the adenovirus infection by downregulation of E1A expression (White *et al.*, 1988) and through interaction with the proapoptotic Bcl-2 family members Bax and Nbk/Bik (Boyd *et al.*, 1994, 1995; Han *et al.*, 1996a,b). Then E1B 19K function may be actively shut down late during infection to allow cytolysis of the host cell and release of viral progeny.

An adenovirus E3-11.6K protein, also termed the adenovirus death protein (ADP), has been proposed to be responsible for cytolysis of adenovirus infected cells at late stages of infection (Tollefson *et al.*, 1996). The possibility arises that ADP may inhibit E1B 19K activity, and possibly the activity of other Bcl-2 family members, late during infection and thereby induce apoptosis to promote virus release. Z-VAD-FMK may inhibit the "late apoptosis" induced by suppression of E1B 19K function, which can account for the reduced virus release. However, ADP has not been shown to induce morphological changes reminiscent of apoptosis (Tollefson *et al.*, 1996).

Thus, the possibility arises that the final cytolysis of adenovirus-infected cells is a pathway distinct from apoptosis, but caspases are involved in both events. In conclusion, Z-VAD-FMK-inhibitable proteases may play a role in modulating the cellular response to adenovirus infection by both inducing apoptosis and enabling virus release.

MATERIALS AND METHODS

Cells, viruses, and viral infections

HeLa cells were maintained in Dulbecco's modified Eagle's medium (DMEM) fortified with 10% fetal bovine serum (FBS). The wild-type Ad5*dl*309 virus and the mutant Ad5*dl*337 virus, which lacks functional E1B 19K due to a deletion in the E1B 19K open reading frame, were used in the experiments and were described earlier (Jones and Shenk, 1979; Pilder *et al.*, 1984). The 12S virus expresses only the 12S E1A gene product and the 12S.337 virus expresses 12S E1A and lacks functional E1B 19K, and they have been described previously (Moran *et al.*, 1986; White *et al.*, 1988; Zerler *et al.*, 1986).

Confluent monolayers of HeLa cells in 60-mm tissue culture plates were infected at a multiplicity of infection (m.o.i.) of 100 plaque-forming units (PFU)/cell with the Ad5*dl*309 or the Ad5*dl*337 virus for the apoptosis inhibition assay (see below). For the virus production and release assays, HeLa cells were infected with the Ad5*dl*309 and Ad5*dl*337 viruses at an m.o.i. of 0.1 PFU/cell, and with the replication-impaired 12S and 12S.337 viruses at an m.o.i. of 100 PFU/cell, since these replication-defective viruses have been shown to grow at high m.o.i. (Moran *et al.*, 1986; White and Stillman, 1987).

Protease inactivation and apoptosis inhibition assays

The peptide Z-VAD-FMK was used in the assays as a general inhibitor of ICE-like caspases. The benzyloxycarbonyl-Phe-Ala-fluoromethyl ketone (Z-FA-FMK) peptide was used as a nonspecific control peptide in the assays. Both were purchased from Enzyme Systems Products (Dublin, CA). Stock concentrations of 100 mM were prepared by dissolving each peptide in dimethyl sulfoxide (DMSO).

At 6 h postinfection, Z-VAD-FMK or Z-FA-FMK was added to the cell culture at a 25 μM concentration. An additional 25 mm of peptide was added to the cell cultures 24 h later due to the short half-life of these peptides. At 48 h postinfection, cells were observed for appearance of the *cyt* phenotype, harvested for Hirt DNA extraction (*deg* phenotype) with a modified Hirt DNA extraction method (White *et al.*, 1984), and harvested for preparation of whole cell extracts for Western blot analysis.

Western blot analysis

Following infection and treatment with peptide inhibitors, whole cell protein extracts were prepared and analyzed for the active p17 cleavage product of CPP32, cleavage products of PARP, which is a known substrate for CPP32, Mch2, Mch3, and Ich-1 (Fernandes-Alnemri *et al.*, 1994, 1995a; Nicholson *et al.*, 1995; Tewari *et al.*, 1995; Wang *et al.*, 1994), and lamins, which are known Mch2 substrates (Orth *et al.*, 1996). The anti-human CPP32-p17 polyclonal antibody (generously provided by D. Nicholson, Merck Frosst, Montreal, Quebec, Canada) directed against the p17 subunit of human CPP32 was used to detect that polypeptide and its cleaved products in infected cells. The monoclonal antibody C2-10 (Enzyme Systems Products) was used to detect PARP and its cleavage products. The 1E4 monoclonal antibody directed against lamin A/C (generously provided by Dr. F. McKeon, Harvard Medical School, Cambridge, MA) was used to detect lamins A and C and their cleavage products.

Virus yield and release assays

For the virus production assay, 25 μm of Z-FA-FMK or Z-VAD-FMK was added at 6 h postinfection and at 24-h intervals thereafter. When infection was complete and cells were rounded and detached from the plate, cells and media were harvested. The control peptide-treated Ad5*dl*309 and Ad5*dl*337 infections were harvested at 48 h postinfection, and the Z-VAD-FMK-treated samples from the same infections were harvested at 72 h postinfection. All the 12S and 12S.337 infections were harvested at 72 h postinfection. For total virus yield, cells plus media were frozen and thawed three times to release the virus particles. The yield of infectious virus was determined by plaque formation on 293 cells.

For the virus release assay, cells and media were separated after harvest by low-speed centrifugation at 1000 rpm for 3 min. The resulting cell pellet was frozen and thawed three times and resuspended in 1 ml of DMEM. Infectious virus yields in the media and in the cell pellet were determined separately by plaque formation on 293 cells. The percent virus release was calculated as the ratio of infectious virus released into the medium to the total infectious virus yield.

ACKNOWLEDGMENTS

We thank F. Mckeon (Harvard Medical School, Cambridge, MA) for generously providing the lamin A/C antibody. This work was supported by NIH Grant CA 60088 to E. White.

REFERENCES

Afonso, C. L., Neilan, J. G., Kutish, G. F., and Rock, D. L. (1996). An african swine fever virus Bcl-2 homolog, 5-HL, suppresses apoptotic cell death. *J. Virol.* 70, 4858–4863.

Ahmad, M., Srinivasula, S. M., Wang, L., Litwack, G., Fernandes-Al-

nemri, T., and Alnemri, E. S. (1997). Spodoptera frugiperda caspase-1, a novel insect death protease that cleaves the nuclear immunophilin FKBP46, is the target of the baculovirus antiapoptotic protein p35. *J. Biol. Chem.* **272**, 1421–1424.

Alnemri, E. S., Livingston, D. J., Nicholson, D. W., Salvesen, G., Thornberry, N. A., Wong, W. W., and Yuan, J. (1996). Human ICE/CED-3 protease nomenclature. *Cell* **87**, 171.

Ankarcrona, M., Zhivotovsky, B., Holmstrom, T., Diana, A., Eriksson, J., Orrenius, S., and Nicotera, P. (1996). Lamin and beta-tubulin fragmentation precede chromatin degradation in glutamate-induced neuronal apoptosis. *Neuroreport* **7**, 2659–2664.

Antoni, B. A., Sabbatini, P., Rabson, A. B., and White, E. (1995). Inhibition of apoptosis in human immunodeficiency virus-infected cells enhances virus production and facilitates persistent infection. *J. Virol.* **69**, 2384–2392.

Berk, A. J. (1986). Adenovirus promoters and E1A transactivation. *Annu. Rev. Genet.* **20**, 5–79.

Berry, D., Lu, Y., Schmidt, B., Fallon, P., O'Connell, C., Hu, S., Xu, H., and Blanck, G. (1996). Retinoblastoma protein inhibits IFN-gamma induced apoptosis. *Oncogene* **12**, 1809–1819.

Boulakia, C. A., Chen, G., Ng, F. W. H., Teodoro, J. G., Branton, P. E., Nicholson, D. W., Poirier, G. G., and Shore, G. C. (1996). Bcl-2 and adenovirus E1B 19 kDa protein prevent E1A-induced processing of CPP32 and cleavage of poly(ADP-ribose) polymerase. *Oncogene* **12**, 529–535.

Boyd, J., Malstrom, S., Subramanian, T., Venkatesh, L., Schaeper, U., Elangovan, B., D'Sa-Eipper, C., and Chinnadurai, G. (1994). Adenovirus E1B 19kDa and bcl-2 proteins interact with a common set of cellular proteins. *Cell* **79**, 341–351.

Boyd, J. M., Gallo, G. J., Elangovan, B., Houghton, A. B., Malstrom, S., Avery, B. J., Ebb, R. G., Subramanian, T., Chittenden, T., Lutz, R. J., and Chinnadurai, G. (1995). Bik1, a novel death-inducing protein shares a distinct sequence motif with Bcl-2 family proteins and interacts with viral and cellular survival-promoting proteins. *Oncogene* **11**, 1921–1928.

Brun, A., Rivas, C., Estaban, M., Escribano, J., and Afonso, C. (1996). African Swine Fever Virus gene A179L, a viral homolog of bcl-2, protects cells from programmed cell death. *Virology* **225**, 227–230.

Bump, N. J., Hackett, M., Hugunin, M., Seshagiri, S., Brady, K., Chen, P., Ferenz, C., Franklin, S., Ghayur, T., Li, P., Licari, P., Mankovich, J., Shi, L., Greenberg, A., Miller, L. K., and Wong, W. W. (1995). Inhibition of ICE family proteases by baculovirus anti-apoptotic protein p35. *Science* **269**, 1885–1888.

Chang, L. F., Killem, K. F., and C. R. Y. (1993). Increased replication of simian immunodeficiency virus in CEM ×174 cells by morphine sulfate. *Biochem. Biophys. Res. Commun.* **195**, 1165–1173.

Cheng, E., Nicholas, J., Bellows, D., Hayward, G., Guo, H., Reitz, M., and Hardwick, J. (1997). A Bcl-2 homolog encoded by Kaposi sarcoma-associated virus, human herpesvirus 8, inhibits apoptosis but does not heterodimerize with Bax or Bak. *Proc. Natl. Acad. Sci. USA* **94**, 690–694.

Chinnaiyan, A. M., Woffendin, C., Dixit, V., and Nabel, G. (1997). The inhibition of pro-apoptotic ICE-like proteases enhances HIV replication. *Nature Med.* **3**, 333–337.

Chiou, S.-K., and White, E. (1997). p300 binding by E1A cosegregates with p53 induction but is dispensable for apoptosis. *J. Virol.* **68**, 6553–6566.

Clem, R. J., and Miller, L. K. (1993). Apoptosis reduces both the in vitro replication and in vivo infectivity of a baculovirus. *J. Virol.* **67**, 3730–3738.

Ellis, H., and Horvitz, H. R. (1986). Genetic control of programmed cell death in the nematode *C. elegans*. *Cell* **44**, 817–829.

Enari, M., Talanian, R. V., Wang, W. W., and Nagata, S. (1996). Sequential activation of ICE-like and CPP32-like proteases during fas-mediated apoptosis. *Nature (London)* **380**, 723–726.

Faucheu, C., Diu, A., Chan, A. W. E., Blanchet, A.-M., Miossec, C., Herve, F., Collard-Dutilleul, V., Gu, Y., Aldape, R. A., Lippke, J. A., Rocher, C., Su, M. S.-S., Livingston, D. J., Hercend, T., and Lalanne, J.-L. (1995). A novel human protease similar to the interleukin-1b converting enzyme induces apoptosis in transfected cells. *EMBO J.* **14**, 1914–1922.

Fernandes-Alnemri, T., Armstrong, R. C., Krebs, J., Srinivasula, S. M., Wang, L., Bullrich, F., Fritz, L. C., Trapani, J. A., Tomaselli, K. J., Litwack, G., and Alnemri, E. S. (1996). In vitro activation of CPP32 and Mch3 by Mch4, a novel human apoptotic cysteine protease containing two FADD-like domains. *Proc. Natl. Acad. Sci. USA* **93**, 7464–7469.

Fernandes-Alnemri, T., Litwack, G., and Alnemri, E. (1994). CPP32, a novel human apoptotic protein with homology to *Caenorhabditis elegans* cell death protein Ced-3 and mammalian interleukin-1b-converting enzyme. *J. Biol. Chem.* **269**, 30761–30764.

Fernandes-Alnemri, T., Litwack, G., and Alnemri, E. S. (1995a). *Mch2*, a new member of the apoptotic *Ced-3/ICE* cysteine protease gene family. *Cancer Res.* **55**, 2737–2742.

Fernandes-Alnemri, T., Takahashi, A., Armstrong, R., Krebs, J., Fritz, L., Tomaselli, K. J., Wang, L., Yu, Z., Croce, C. M., Salveson, G., Earnshaw, W. C., Litwack, G., and Alnemri, E. S. (1995b). Mch3, a novel human apoptotic cysteine protease highly related to CPP32. *Cancer Res.* **55**, 6045–6052.

Fraser, A., and Evan, G. (1996). A license to kill. *Cell* **85**, 781–784.

Fraser, A., and Evan, G. (1997). Identification of a *Drosophila melanogaster* ICE/Ced-3-related protease, drICE. *EMBO J.* **16**, 2805–2813.

Gooding, L. R. (1992). Virus proteins that counteract host immune defenses. *Cell* **71**, 5–7.

Haas-Kogan, D. A., Kogan, S. C., Levi, D., Dazin, P., T'Ang, A., Fung, Y.-K., and Israel, M. A. (1995). Inhibition of apoptosis by the retinoblastoma gene product. *EMBO J.* **14**, 461–472.

Han, J., Sabbatini, P., Perez, D., Rao, L., Mohda, D., and White, E. (1996a). The E1B 19K protein blocks apoptosis by interacting with and inhibiting the p53-inducible and death-promoting Bax protein. *Genes Dev.* **10**, 461–477.

Han, J., Sabbatini, P., and White, E. (1996b). Induction of apoptosis by human Nbk/Bik, a BH3 containing E1B 19K interacting protein. *Mol. Cell. Biol.* **16**, 5857–5864.

Harvey, A., Bidwai, A., and Miller, L. (1997). Doom, a product of the *Drosophila* mod(mdg4) gene, induces apoptosis and binds to baculovirus inhibitor-of-apoptosis proteins. *Mol. Cell. Biol.* **17**, 2835–2843.

Hengartner, M. O., and Horvitz, H. R. (1994). Programmed cell death in *Caenorhabditis elegans*. *Curr. Opin. Genet. Dev.* **4**, 581.

Howes, K. A., Ransom, N., Papermaster, D. S., Lasudry, J. G. H., Albert, D. M., and Windle, J. J. (1994). Apoptosis or retinoblastoma: Alternative fates of photoreceptors expressing the HPV-16 E7 gene in the presence or absence of p53. *Genes Dev.* **8**, 1300–1310.

Janicke, R., Walker, P., Xiao, Y., and Porter, A. (1996). Specific cleavage of the retinoblastoma protein by an ICE-like protease in apoptosis. *EMBO J.* **15**, 6969–6978.

Jones, N., and Shenk, T. (1979). An adenovirus type 5 early gene function regulates expression of other viral genes. *Proc. Natl. Acad. Sci. USA* **76**, 3665–3669.

Kamens, J., Paskind, M., Huguinin, M., Talanian, R. V., Allen, H., Banach, D., Bump, N., Hackett, M., Johnston, C. G., Li, P., Mankovitch, J. A., Terranova, M., and Ghayur, T. (1995). Identification and characterization of Ich-2, a novel member of the interleukin-1b-converting enzyme family of cysteine proteases. *J. Biol. Chem.* **270**, 15250–15256.

Kastan, M. B., Onyekwere, O., Sidransky, D., Vogelstein, B., and Craig, R. W. (1991). Participation of p53 protein in the cellular response to DNA damage. *Cancer Res.* **51**, 6304–6311.

Komiyama, T., Ray, C. A., Pickup, D. J., Howard, A. D., Thornberry, N. A., Peterson, E. P., and Salvesen, G. (1994). Inhibition of interleukin-1b converting enzyme by the cowpox virus serpin CrmA. *J. Biol. Chem.* **269**, 19331–19337.

Kuida, K., Zheng, T. S., Na, S., Kuan, C.-Y., Yang, D., Karasuyama, H., Rakic, P., and Flavell, R. A. (1996). Decreased apoptosis in the brain and premature lethality in CPP32-deficient mice. *Nature (London)* **384**, 368–372.

Kumar, S. (1995). ICE-like proteases in apoptosis. *Trends Biochem. Sci.* **20**, 198–202.

Lazebnik, Y. A., Kaufmann, S. H., Desnoyers, S., Poirier, G. G., and Earnshaw, W. C. (1994). Cleavage of poly(ADP-ribose) polymerase by a proteinase with properties like ICE. *Nature (London)* **371**, 346–347.

Leopardi, R., and Roizman, B. (1996). The herpes simplex virus major regulatory protein ICP4 blocks apoptosis induced by the virus or by hyperthermia. *Proc. Natl. Acad. Sci. USA* **93**, 9583–9587.

Lillie, J. W., Loewenstein, P. M., Green, M. R., and Green, M. (1987). Functional domains of adenovirus type 5 E1a proteins. *Cell* **50**, 1091–1100.

Lippke, J. A., Yong, G., Sarnecki, C., Caron, P. R., and Su, M. S.-S. (1995). Identification and characterization of CPP32/Mch2 homolog 1, a novel cysteine protease similar to CPP32. *J. Biol. Chem.* **271**, 1825–1828.

Marcellus, R. C., Teodoro, J. G., Charbonneau, R., Shore, G. C., and Branton, P. E. (1996). Expression of p53 in Saos-2 osteosarcoma cells induces apoptosis which can be inhibited by Bcl-2 or the adenovirus E1B 55 kDa protein. *Cell Growth Differ.* **7**, 1643–1650.

Martin, S. J., and Green, D. R. (1995). Protease activation during apoptosis: Death by a thousand cuts? *Cell* **82**, 349–352.

McCarthy, N. J., Whyte, M. K. B., Gilbert, C. S., and Evan, G. I. (1997). Inhibition of Ced-3/ICE-related proteases does not prevent cell death induced by oncogenes, DNA damage, or the Bcl-2 homologue Bak. *J. Cell. Biol.* **136**, 215–227.

McConkey, D., Goodrich, D., Bucana, C., and Klostergaard, J. (1996). The human retinoblastoma gene product suppresses ceramide-induced apoptosis in human bladder tumor cells. *Oncogene* **13**, 1693–1700.

Moran, E. (1993). DNA tumor virus transforming proteins and the cell cycle. *Curr. Opin. Genet. Dev.* **3**, 63–70.

Moran, E., Grodzicker, T., Roberts, R. J., Mathews, M. B., and Zerler, B. (1986). Lytic and transforming functions of individual products of the adenovirus E1A gene. *J. Virol.* **57**, 765–775.

Moran, E., and Mathews, M. B. (1987). Multiple functional domains in the adenovirus E1A gene. *Cell* **48**, 177–178.

Munday, N. A., Vaillancourt, J. P., Ali, A., Casano, F. J., Miller, D. K., Molineaux, S. M., Yamin, T.-T., Yu, V. L., and Nicholson, D. W. (1995). Molecular cloning and pro-apoptotic activity of ICErel-II and ICErel-III, members of the ICE/CED-3 family of cysteine proteases. *J. Biol. Chem.* **270**, 15870–15876.

Nicholson, D. W. (1996). ICE/CED3-like proteases as therapeutic targets for the control of inappropriate apoptosis. *Nature Biotech.* **14**, 297–301.

Nicholson, D. W., Ali, A., Thornberry, N. A., Vaillancourt, J. P., Ding, C. K., Gallant, M., Gareau, Y., Griffin, P. R., Labelle, M., Lazebnik, Y. A., Munday, N. A., Raju, S. M., Smulson, M. E., Yamin, T.-T., Yu, V. L., and Miller, D. K. (1995). Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. *Nature (London)* **376**, 37–43.

Oberhammer, F. A., Hochegger, K., Froschl, G., Tiefenbacher, R., and Pavelka, M. (1994). Chromatin condensation during apoptosis is accompanied by degradation of lamin A + B, without enhanced activation of cdc2 kinase. *J. Cell Biol.* **126**, 827–837.

Orth, K., Chinnaiyan, A. M., Garg, M., Froelich, C. J., and Dixit, V. M. (1996). The CED-3/ICE-like protease Mch2 is activated during apoptosis and cleaves the death substrate lamin A. *J. Biol. Chem.* **271**, 16443–16446.

Pilder, S., Logan, J., and Shenk, T. (1984). Deletion of the gene encoding the adenovirus 5 early region 1B - 21,000-molecular weight polypeptide leads to degradation of viral and cellular DNA. *J. Virol.* **52**, 664–671.

Rao, L., Modha, D., and White, E. (1997). The E1B 19K protein associates with lamins in vivo and its proper localization is required for inhibition of apoptosis. *Oncogene* **15**, 1587–1597.

Rao, L., Perez, D., and White, E. (1996). Lamin proteolysis facilitates nuclear events during apoptosis. *J. Cell Biol.* **135**, 1441–1455.

Rao, L., and White, E. (1997). Bcl-2 and ICE family of apoptotic regulators: Making a connection. *Curr. Opin. Genet. Dev.* **7**, 52–58.

Sabbatini, P., Han, J. H., Chiou, S.-K., Nicholson, D., and White, E. (1997). ICE-like proteases are essential for p53-mediated transcriptionally-dependent apoptosis. *Cell Growth Differ.* **8**, 643–653.

Sandstrom, P., Pardi, D., Goldsmith, C., Chengying, D., Diamond, A., and Folks, T. (1996). bcl-2 expression facilitates human immunodeficiency virus type-1 mediated cytopathic effects during acute spreading infections. *J. Virol.* **70**, 4617–4622.

Song, Z., McCall, K., and Steller, H. (1997). DCP-1, a *Drosophila* cell death protease essential for development. *Science* **275**, 536–540.

Takahashi, A., Alnemri, E. S., Lazebnik, Y. A., Fernandes-Alnemri, T., Litwack, G., Moir, R. D., Goldman, R. D., Poirier, G. G., Kaufmann, S. H., and Earnshaw, W. C. (1996). Cleavage of lamin A by Mch2a but not CPP32: Multiple ICE-related proteases with distinct substrate recognition properties are active in apoptosis. *Proc. Natl. Acad. Sci. USA* **93**, 8395–8400.

Tan, X., Seamus, J., Green, D., and Wang, J. (1997). Degradation of retinoblastoma protein in tumor necrosis factor- and CD95-induced cell death. *J. Biol. Chem.* **272**, 9613–9616.

Teodoro, J. G., Shore, G. C., and Branton, P. E. (1995). Adenovirus E1A proteins induce apoptosis by both p53-dependent and p53-independent mechanisms. *Oncogene* **11**, 467–474.

Tewari, M., and Dixit, V. M. (1995). Fas- and TNF-induced apoptosis is inhibited by the poxvirus *crmA* gene product. *J. Biol. Chem.* **270**, 3255–3260.

Tewari, M., Quan, L. T., O'Rourke, K., Desnoyers, S., Zeng, Z., Beidler, D. R., Poirier, G., Salvesen, G. S., and Dixit, V. M. (1995). Yama/CPP32b, a mammalian homolog of CED-3, is a CrmA-inhibitable protease that cleaves the death substrate poly(ADP-ribose) polymerase. *Cell* **81**, 801–809.

Tollefson, A. E., Scaria, A., Hermiston, T. W., Ryerse, J. S., Wold, L. J., and Wold, W. S. M. (1996). The adenovirus death protein (E3-11.6K) Is required at very late stages of infection for efficient cell lysis and release of adenovirus from infected cells. *J. Virol.* **70**, 2296–2306.

Ubol, S., Park, S., Budihardjo, I., Desnoyers, S., Montrose, M. H., Poirier, G., Kaufmann, S., and Griffin, D. (1996). Temporal changes in chromatin, intracellular calcium, and poly(ADP-ribose) polymerase during sinbis virus-induced apoptosis of neuroblastoma cells. *J. Virol.* **70**, 2215–2220.

Waga, S., Hannon, G. J., Beach, D., and Stillman, B. (1994). The p21 inhibitor of cyclin-dependent kinases controls DNA replication by interaction with PCNA. *Nature (London)* **369**, 574–578.

Wang, L., Miura, M., Bergeron, L., Zhu, H., and Yuan, J. (1994). *Ich-1*, an *ICE/ced-3*-related gene, encodes both positive and negative regulators of programmed cell death. *Cell* **78**, 739–750.

Wang, Z.-Q., Auer, B., Stingl, L., Berghammer, H., Haidacher, D., Schweiger, M., and Wagner, E. F. (1995). Mice lacking ADPRT and poly(ADP-ribosyl)ation develop normally but are susceptible to skin disease. *Genes Dev.* **9**, 509–520.

White, E. (1993). Regulation of apoptosis by the transforming genes of the DNA tumor virus adenovirus. *Proc. Soc. Exp. Biol. Med.* **204**, 30–39.

White, E., Cipriani, R., Sabbatini, P., and Denton, A. (1991). The adenovirus E1B 19-Kilodalton protein overcomes the cytotoxicity of E1A proteins. *J. Virol.* **65**, 2968–2978.

White, E., Denton, A., and Stillman, B. (1988). Role of the adenovirus E1B 19,000-dalton tumor antigen in regulating early gene expression. *J. Virol.* **62**, 3445–3454.

White, E., and Gooding, L. R. (1994). Regulation of apoptosis by human adenoviruses. *In* "Apoptosis: The Molecular Basis for Cell Death (D. T. and F. Cope, Eds.), Vol. II, pp. 111–141. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

White, E., Grodzicker, T., and Stillman, B. W. (1984). Mutations in the gene encoding the adenovirus E1B 19K tumor antigen cause degradation of chromosomal DNA. *J. Virol.* **52**, 410–419.

White, E., Sabbatini, P., Debbas, M., Wold, W. S. M., Kusher, D. I., and Gooding, L. (1992). The 19-kilodalton adenovirus E1B transforming protein inhibits programmed cell death and prevents cytolysis by tumor necrosis factor a. *Mol. Cell. Biol.* 12, 2570–2580.

White, E., and Stillman, B. (1987). Expression of the adenovirus E1B mutant phenotypes is dependent on the host cell and on synthesis of E1A proteins. *J. Virol.* 61, 426–435.

Xue, D., and Horvitz, H. R. (1995). Inhibition of the *Ceanorhabditis elegans* cell-death protease CED-3 by a CED-3 cleavage site in baculovirus p35 protein. *Nature (London)* 377, 248–251.

Xue, D., Shaham, S., and Horvitz, H. R. (1996). The *Caenorhabditis elegans* cell-death protein CED-3 is a cysteine protease with substract specificities similar to those of the human CPP32 protease. *Genes Dev.* 10, 1073–1083.

Yuan, J., Shaham, S., Ledoux, S., Ellis, H. M., and Horvitz, H. R. (1993). The *C. elegans* cell death gene ced-3 encodes a protein similar to mammalian interleukin-1b-converting enzyme. *Cell* 75, 641–652.

Zerler, B., Moran, E., Maruyama, K., Moomaw, J., Grodzicker, T., and Ruley, H. E. (1986). Adenovirus E1A coding sequences that enable ras and pmt oncogenes to transform cultured primary cells. *Mol. Cell. Biol.* 6, 887–889.

Zhu, H., Fearnhead, H. O., and Cohen, G. M. (1995). An ICE-like protease is a common mediator of apoptosis induced by diverse stimuli in human monocytic THP.1 cells. *FEBS Lett.* 374, 303–308.

EXHIBIT B

JOURNAL OF VIROLOGY, Oct. 2001, p. 8875–8887
0022-538X/01/$04.00+0 DOI: 10.1128/JVI.75.19.8875–8887.2001

Vol. 75, No. 19

Inhibition of TRAIL-Induced Apoptosis and Forced Internalization of TRAIL Receptor 1 by Adenovirus Proteins

ANN E. TOLLEFSON,[1] KAROLY TOTH,[1] KONSTANTIN DORONIN,[1] MOHAN KUPPUSWAMY,[1] OKSANA A. DORONINA,[1] DREW L. LICHTENSTEIN,[1] TERRY W. HERMISTON,[1] CRAIG A. SMITH,[2] AND WILLIAM S. M. WOLD[1]*

Department of Molecular Microbiology and Immunology, St. Louis University Health Sciences Center, St. Louis, Missouri 63104,[1] and Immunex Corporation, Seattle, Washington 98101[2]

Received 10 January 2001/Accepted 4 June 2001

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) induces apoptosis through two receptors, TRAIL-R1 (also known as death receptor 4) and TRAIL-R2 (also known as death receptor 5), that are members of the TNF receptor superfamily of death domain-containing receptors. We show that human adenovirus type 5 encodes three proteins, named RID (previously named E3-10.4K/14.5K), E3-14.7K, and E1B-19K, that independently inhibit TRAIL-induced apoptosis of infected human cells. This conclusion was derived from studies using wild-type adenovirus, adenovirus replication-competent mutants that lack one or more of the *RID*, *E3-14.7K*, and *E1B-19K* genes, and adenovirus E1-minus replication-defective vectors that express all E3 genes, RID plus E3-14.7K only, RID only, or E3-14.7K only. RID inhibits TRAIL-induced apoptosis when cells are sensitized to TRAIL either by adenovirus infection or treatment with cycloheximide. RID induces the internalization of TRAIL-R1 from the cell surface, as shown by flow cytometry and indirect immunofluorescence for TRAIL-R1. TRAIL-R1 was internalized in distinct vesicles which are very likely to be endosomes and lysosomes. TRAIL-R1 is degraded, as indicated by the disappearance of the TRAIL-R1 immunofluorescence signal. Degradation was inhibited by bafilomycin A1, a drug that prevents acidification of vesicles and the sorting of receptors from late endosomes to lysosomes, implying that degradation occurs in lysosomes. RID was also shown previously to internalize and degrade another death domain receptor, Fas, and to prevent apoptosis through Fas and the TNF receptor. RID was shown previously to force the internalization and degradation of the epidermal growth factor receptor. E1B-19K was shown previously to block apoptosis through Fas, and both E1B-19K and E3-14.7K were found to prevent apoptosis through the TNF receptor. These findings suggest that the receptors for TRAIL, Fas ligand, and TNF play a role in limiting virus infections. The ability of adenovirus to inhibit killing through these receptors may prolong acute and persistent infections.

Adenovirus (Ad) has been widely studied as a model for virus replication, gene regulation, oncogenic cell transformation, and immune evasion. Ad infection in cell culture proceeds in well-regulated phases. The immediate-early E1A proteins, derived from the E1A transcription unit, induce transcription of delayed-early genes in the E1B, E2, E3, and E4 transcription units. Viral DNA begins to replicate at about 7 h postinfection (p.i.), and then late, primarily structural genes are expressed. Virions begin to assemble in the cell nucleus at about 1 day p.i. The cells begin to lyse at 2 to 3 days p.i. and release virus particles.

It is important that the infected cell remain intact during this extended period of infection. Indeed, Ads have evolved proteins that protect infected cells against apoptosis induced by cells and agents of the immune system (reviewed in references 14, 49, 69, 83, 87, 89, and 90). Most of these Ad proteins are encoded by the E3 and E1B transcription units. One such protein, named E3-gp19K, is a membrane glycoprotein localized in the endoplasmic reticulum. E3-gp19K forms a complex with major histocompatibility complex class I antigens, blocks their transport to the cell surface, and prevents killing of infected cells by cytotoxic T lymphocytes (CTL). Three Ad proteins inhibit apoptosis induced by tumor necrosis factor alpha (TNF-α) and Fas ligand (FasL; also known as CD95L). These ligands are expressed on activated leukocytes and are also shed in functional form; interact with their cognate receptors, TNF receptor 1 (TNFR1) and Fas (also known as CD95 and ApoI), respectively; and induce apoptosis by activation of caspases. The E3 protein named RID (for receptor internalization and degradation), a complex of the RIDα and RIDβ proteins (formerly known as E3-10.4K and E3-14.5K), is an integral membrane protein localized primarily on the cell surface (34, 67, 74, 75). RID inhibits apoptosis through the Fas pathway (19, 65, 72) by stimulating the internalization of cell surface Fas into endosomes, which are transported to lysosomes, where Fas is degraded (72). RID also inhibits TNF-induced apoptosis (23, 42). Another E3 protein, a nonmembrane protein named E3-14.7K (78), independently inhibits TNF-induced apoptosis (22, 24, 42). E3-14.7K is also reported to inhibit apoptosis induced through the Fas pathway (13). Finally, the protein named E1B-19K inhibits apoptosis induced through the TNF and Fas pathways (21, 31, 56, 72, 84).

TNF and FasL are members of the TNF superfamily.

* Corresponding author. Mailing address: Department of Molecular Microbiology and Immunology, St. Louis University Health Sciences Center, 1402 S. Grand Blvd., St. Louis, MO 63104. Phone: (314) 577-8435. Fax: (314) 773-3403. E-mail: woldws@slu.edu.

TNFR1 and Fas are members of the TNFR superfamily and contain "death domains" (reviewed in references 28, 53, 61, and 62). Death domains are conserved protein domains that participate in protein-protein interactions leading to activation of caspases that mediate apoptosis. TNF-related apoptosis-inducing ligand (TRAIL [also known as Apo2L]) is another member of the TNF superfamily that induces apoptosis (51, 58, 85), and two of the TRAIL receptors, TRAIL-R1 (also known as death receptor 4) and TRAIL-R2 (also known as death receptor 5), contain death domains (12, 54, 55, 64, 81). TRAIL and its receptors are expressed on many cell types (25).

TRAIL and the TRAIL receptors have been shown to play a role in a number of viral infections. T cells from human immunodeficiency virus-infected patients are killed by TRAIL (35, 38). Human cytomegalovirus (CMV) infection of primary human fibroblasts increased cell surface expression of TRAIL-R1 and TRAIL-R2, and TRAIL displayed potent antiviral activity in vitro on human CMV-infected fibroblasts (63). TRAIL and TRAIL receptors contribute to the apoptosis and pathology associated with reovirus infection (16) and are suggested to be involved in immunosuppression observed with measles infection (80).

TRAIL is emerging as another molecule used by cells of the immune system to kill virus-infected and tumor cells. Reports indicate that activated T and B cells express TRAIL (50, 52) and that TRAIL mediates killing by $CD4^+$ CTL (39, 70). Several groups have found that human natural killer (NK) cells express TRAIL (37) and show TRAIL-dependent cytotoxicity (35, 37). TRAIL and TRAIL receptor expression could also be induced in a number of cells by interferon (IFN) treatment. IFN-γ and TNF induced TRAIL expression in primary human fibroblasts (63). Type I IFNs induced TRAIL expression on both $CD4^+$ and $CD8^+$ peripheral blood T cells (40). After IFN-γ or IFN-α treatment, human monocytes (27) and dendritic cells (20) expressed TRAIL and were able to kill tumor cells. Following treatment of monocytes with Type I IFNs, monocytes developed into TRAIL-expressing dendritic cells, which showed antiviral and antitumor effects (62). Thus, it might be expected that the TRAIL pathway would be targeted for inactivation by adenoviruses.

In this study we show that the Ad RID, E3-14.7K, and E1B-19K proteins independently inhibit TRAIL-induced apoptosis. As is the case with Fas, RID stimulates the internalization and degradation of TRAIL-R1.

MATERIALS AND METHODS

Cell lines. Human A549 lung carcinoma cells (American Type Culture Collection [ATCC]), human 293 cells, and human HeLa cervical carcinoma cells were grown in Dulbecco's modified essential medium containing 10% fetal bovine serum. HT29.14S cells (10) (received from Jeff Browning, Biogen, Inc., Cambridge, Mass.; a clone derived from the HT29 colon carcinoma cell line [ATCC]) were grown in McCoy's medium with 10% fetal bovine serum. The E3-14.7K-expressing lines 4-6-8F and 37-2-1-2B were derived from A549 cells stably cotransfected with plasmids pSV2neo and either pMT2-14.7K or pMT2-14.7K(D37A) and selected in G418. The cell lines were isolated following subcloning of individual colonies. Clone 4-6-8F expresses the wild-type (WT) Ad5 E3-14.7K protein; clone 37-2-1-2B expresses an E3-14.7K protein in which a point mutation (D37 changed to A37) has been built into E3-14.7K (this has been shown not to alter E3-14.7K function).

Viruses. Viruses used in these studies include Ad type 5 (Ad5), *dl*309 (Ad5 derivative; RID^- 14,700-molecular-weight protein [$14.7K]^-$) (36), *dl*111 (Ad5 derivative; RID^- $14.7K^-$ $E1B\text{-}19K^-$) (3), *lp*5 (Ad2 derivative with a point mutation in E1B-19K) (68), *dl*250 (Ad2 derivative; $E1B\text{-}19K^-$) (68), *rec*700 (the WT parental virus for viruses with 700 numbers; an Ad5-Ad2-Ad5 recombinant) (88), *dl*758 ($14.7K^-$) (8), *dl*7000 (Ad5 derivative expressing only 14.7K from E3) (59), *dl*701 ($6.7K^-$) (6), *dl*754 (deletion and modification of E3-6.7K C terminus and deletion of E3-gp19K) (22), *dl*704 (E3-$gp19K^-$) (6), *dl*731 (E3-$12.5K^-$) (8), *pm*734.1 (double point mutations eliminate the first two methionine codons of the Ad death protein [ADP]; ADP is not expressed) (76), *dl*762 ($14.7K^-$) (8), *dl*764 ($RID\beta^-$) (74), *dl*799 (RID^-) (23), and *pm*760 (increased expression of RID; decreased expression of 14.7K) (9). Table 1 provides additional information. Virus stocks were grown in KB suspension cultures, and virus titers were determined by plaque assay on A549 cells as described previously(73).

TABLE 1. Viruses used in this study[a]

Virus[b]	RIDα	RIDβ	E3-14.7K	E1B-19K
*rec*700	+	+	+	+
Ad5	+	+	+	+
*dl*111	—	—	—	—
*dl*309	—	—	—	+
*lp*5	+	+	+	—
*dl*250	+	+	+	—
*dl*764	+	—	+	+
*dl*799	—	—	+	+
*dl*758	+	+	—	+
*dl*762	+	+	—	+
*pm*760	++	++	±	+
*dl*7000	—	—	+	+
*dl*701	+	+	+	+
*dl*704	+	+	+	+
*dl*731	+	+	+	+
*pm*734.1	+	+	+	+
Ad/E3	+	+	+	—
Ad/RID/14.7K	+	+	+	—
Ad/RID	+	+	—	—
Ad/14.7K	—	—	+	—

[a] All mutants except Ad/E3, Ad/RID/14.7K, Ad/RID, and Ad/14.7K are fully competent for replication in cultured cells. Ad/E3 is an $E1^-$ replication-defective Ad vector expressing all E3 Ad proteins except ADP from the CMV promoter. E3 e-encoded proteins are named RIDα, RIDβ, 14.7K, gp19K, 6.7K, 12.5K, and ADP. RIDα and RIDβ function together as the protein complex named RID. Ad/RID/14.7K is an $E1^-$ vector expressing only RID and 14.7K. Ad/RID is an $E1^-$ vector expressing only RID. Ad/14.7K is an $E1^-$ vector expressing only 14.7K. +, expressed; −, not expressed; ++, overexpressed; ±, underexpressed.

[b] *rec*700 is an Ad5-Ad2-Ad5 recombinant and is the WT parent for mutants with 700 or 7000 numbers. Ad5 is the WT parent for *dl*111 and *dl*309. *lp*5 and *dl*250 are derived from Ad2, which is very closely related to Ad5 and *rec*700. *dl*701 lacks the gene for the E3-6.7K protein. *dl*704 lacks the gene for the E3-gp19K protein. *dl*731 lacks the gene for the E3-12.5K protein. *pm*734.1 lacks the gene for ADP (previously named E3-11.6K).

Ad vectors. Ad/E3, Ad/RID/14.7K, Ad/14.7K, Ad/RID, and Ad/null are replication-deficient Ad vectors and were constructed according to the method described by Bett et al. (5); the construction will be described in detail elsewhere. Briefly, the E3 transcription unit of *pm*734.1 (76) was cloned into pcDNA3.1Zeo(+) (Invitrogen, Carlsbad, Calif.). Ad *pm*734.1 contains point mutations in the Met_1 and Met_{41} codons of the *adp* gene and therefore does not express functional ADP. The whole expression cassette (CMV promoter, intron, and E3 genes) was excised and cloned into pdlE1sp1A (Microbix, Toronto, Canada), resulting in plasmid p231. p231 (the precursor plasmid for Ad/E3) expresses all E3 proteins except ADP, as shown by immunoblotting and immunofluorescence. pOD1 is very similar to p231, except it has the genes for the E3-12.5K, E3-6.7K, and E3-gp19K proteins deleted; this plasmid was used for construction of Ad/RID/14.7K. pOD2 and pOD3 express only the 14.7K or the RID protein, respectively, and were used to construct Ad/14.7K and Ad/RID, respectively. p371 has all the E3 genes deleted but retains the CMV promoter; it was used to produce the control Ad/null (empty) vector. These plasmids were sequenced to verify the inserts and then were cotransfected with pBHG10 (Microbix) into 293 cells. The viruses that resulted from recombinations were plaque purified three times on 293 cells, analyzed by DNA digestion using *Hin*dIII, and tested for the absence or presence of specific E3 proteins by immunoblotting, indirect immunofluorescence, and immunoprecipitation (data not shown). The viruses were grown in 293 suspension cultures and purified by CsCl banding. Titers were determined by plaque assay on 293 cells.

Antibodies. A mouse monoclonal antibody (MAb) specific for TRAIL-R1 (M271) (26) was obtained from Immunex Corp. (Seattle, Wash.). Antibodies to transferrin receptor (TfnR) (OKT9) and Fas (M38) were from hybridoma cell lines obtained from ATCC. Antibody to the epidermal growth factor receptor (EGFR) (528) was purchased from Santa Cruz Biotechnology (Santa Cruz, Calif.). Rabbit polyclonal anti-peptide antibody to the Ad DNA binding protein (DBP) was a gift from Maurice Green (48). Rabbit polyclonal anti-peptide antibody to RIDβ has been described previously (74). Fluorochrome-conjugated, affinity-purified secondary antibodies were purchased from Cappel/ICN (Costa Mesa, Calif.).

Phase microscopy. A549 cells were infected with 100 PFU (or 10 PFU/cell for *dl*111) of the indicated viruses/cell. At 14 h p.i., the cells were treated with TRAIL (200 ng/ml) in medium containing 25 μg of cycloheximide (CHX)/ml. After 7 h of TRAIL treatment, the cells were photographed on Tmax 400 film on a Nikon TMS inverted microscope.

Indirect immunofluorescence. A549 or HeLa cells were plated on glass coverslips. The cells were infected with 50 to 400 PFU of virus/cell (as indicated in the figure legends). Some cells were treated with bafilomycin A1 (Baf) (0.1 μM) to inhibit acidification of lysosomes and to disrupt lysosomal degradation of internalized proteins (79, 91). The cells were fixed at the times indicated in the figure legends. For EGFR and RIDβ staining, cells were fixed in methanol (−20°C) containing 4′,6-diamidino-2-phenylindole (DAPI) for 10 min. For TRAIL-R1 or DBP staining, cells were fixed in 3.7% paraformaldehyde for 10 min at room temperature and then permeabilized with methanol (−20°C) containing DAPI for 6 min. The cells were rehydrated with three washes of phosphate-buffered saline and then were stained with antiserum to TRAIL-R1 (M271) at a concentration of 10 μg/ml. EGFR MAb (528) was diluted to 1 μg/ml; DBP and RIDβ antisera were used at 1:400 and 1:250 dilutions, respectively. All antibodies were diluted in phosphate-buffered saline containing 1% bovine serum albumin and 0.1% sodium azide. The secondary antibodies were affinity-purified goat anti-mouse immunoglobulin G (IgG)-fluorescein isothiocyanate (FITC) conjugate for the MAb and goat anti-rabbit IgG-FITC conjugate for the rabbit polyclonal antibodies (Cappel/ICN). The mounting medium contained *p*-phenylenediamine as an antifading agent. The cells were photographed on Tmax 400 film on a Nikon Optiphot microscope equipped with epifluorescence. The film was developed in Diafine developer (Accufine) and fixed in Kodak fixer.

Apoptosis assays. Apoptosis assays were conducted at 1 to 2 days p.i., a period well before these assays detect virus-induced cytotoxicity. Cells were infected with 100 PFU of the E1B-19K-positive replication-competent viruses/cell. For the E1B-19K-negative viruses *dl*111, *dl*250, and *lp5*, 5 to 25 PFU/cell was used (to reduce the cytolytic phenotype of these mutants). To confirm that cells were well infected, an immunofluorescence assay of DBP expression was quantitated at approximately 24 h p.i. For the E1-minus replication-defective vectors, 5 to 20 PFU/cell was used. For all viruses and vectors, cells at 4 to 5 h p.i. were trypsinized, diluted, and plated into 96-well plates. At approximately 24 h p.i., the cells were treated with serial dilutions of TRAIL (0.5 to 50 ng of leucine zipper TRAIL/ml; received from Immunex) (82) in medium containing 25 μg of CHX/ml. After approximately 24 h of TRAIL treatment, the supernatants were removed from the wells and assayed for lactate dehydrogenase (LDH) release using the CytoTox96 assay (Promega, Madison, Wis.). The following equation was used: percent specific lysis = (absorbance with TRAIL − absorbance with CHX)/(maximum absorbance − absorbance with CHX) × 100. The E3-14.7K stably transfected cell lines and their parental A549 cells were treated with 1 ng of TRAIL/ml for 25 h, and then trypan blue exclusion was used to determine the percentage of viable cells.

In some experiments, cell viability was assayed in parallel with the CellTiter 96 Aqueous One Solution cell proliferation assay (Promega). MTS (Owen's reagent) is bioreduced to a colored formazan product, which is soluble in the tissue culture medium and is measured at 490 nm in the original tissue culture plate.

Flow cytometry. Cells were infected at 100 to 150 PFU of replication-competent viruses/cell or 5 to 20 PFU of E1-minus replication-defective viruses/cell, and staining was begun at 23 to 24 h p.i. as indicated in the figure legends. Live cells were incubated on ice with mouse monoclonal primary antibodies in fluorescence-activated cell sorter buffer at the following concentrations: TRAIL-R1 (M271), 5 μg/ml; Fas MAb (M38), 1:4 dilution of culture supernatant; EGFR MAb (Santa Cruz 528), 1 μg/ml; TfnR MAb (OKT9), 1:4 dilution of culture supernatant. The secondary antibody was affinity-purified goat anti-mouse IgG-FITC conjugate (whole molecule; Cappel/ICN). The cells were analyzed on a FACScaliber flow cytometer using Cell Quest software. The figures are presented as three-dimensional overlays of the flow cytometry data. Each curve represents data from 10,000 gated events.

RESULTS

RID, E3-14.7K, or E1B-19K protein is required to inhibit TRAIL-induced apoptosis in Ad-infected cells. Human A549 cells were mock infected or infected with WT Ad (named *rec*700). At 19 h p.i., the cells were treated for 26 h with TRAIL (20 ng/ml) plus CHX (25 μg/ml). CHX was used because it increases the sensitivity of cells to apoptosis induced by TRAIL (44) and TNF (41, 44, 53). The cells were fixed, permeabilized with methanol containing DAPI (to stain nuclei), and then immunostained for the Ad-coded DBP. With mock-infected cells, nuclei were apoptotic, i.e., they were shrunken, the DNA was condensed, and apoptotic bodies were apparent (Fig. 1A). Cells infected with WT Ad, indicated by the speckled staining pattern for DBP in the cell nucleus, had nonapoptotic nuclei (Fig. 1B). We conclude that TRAIL induces apoptosis in A549 cells and that this apoptosis is inhibited by Ad infection.

Virus mutants lacking combinations of the RID, E3-14.7K, and E1B-19K proteins (Table 1), previously shown to inhibit TNF- and Fas agonist-induced apoptosis, were tested to determine whether these viral proteins also inhibit TRAIL-induced apoptosis. A549 cells were infected, treated with TRAIL plus CHX, and then examined by phase-contrast microscopy. Mock-infected cells were apoptotic, but WT Ad-infected cells remained flat, attached, and viable (Fig. 2A and B). With a mutant lacking RID and E3-14.7K but expressing E1B-19K (i.e., RID^- $14.7K^-$ $E1B^+$) (*dl*309) or a mutant expressing RID and E3-14.7K but not E1B-19K (RID^+ $14.7K^+$ $E1B\text{-}19K^-$) (*lp5*), most of the cells remained viable (Fig. 2C and D). However, with a RID^- $14.7K^-$ $E1B\text{-}19K^-$ mutant (*dl*111), the cells were killed by TRAIL plus CHX (Fig. 2E) but not by CHX alone (Fig. 2F). These results indicate that Ad has at least two independent functions that inhibit TRAIL-induced apoptosis, one being E1B-19K and the other being RID, E3-14.7K, or both E3 proteins.

TRAIL-induced apoptosis was further examined by release of LDH from A549 cells, and additional virus mutants were tested (Table 1). TRAIL plus CHX induced apoptosis in mock-infected cells, and this was inhibited by WT Ad (*rec*700 or Ad5) but not by a RID^- $14.7K^-$ $E1B\text{-}19K^-$ mutant (*dl*111), as indicated by three independent assays: LDH release (Fig. 3A), trypan blue exclusion (data not shown), and the MTS assay for mitochondrial activity (data not shown). Similar LDH release results were obtained with HeLa cells (Fig. 3B). Parallel immunofluorescence studies indicated that nearly all the *dl*111-infected cells were expressing DBP (i.e., the cells were well infected) and that the DBP-positive cells had apoptotic nuclei (data not shown). TRAIL-induced killing was prevented by any mutant that expresses at least one of the E1B-19K, RID, or 14.7K proteins; the data are represented by the overlapping curves near the bottom of Fig. 3A and B. For example, TRAIL-induced apoptosis was blocked by mutants with the following genotypes: RID^+ $14.7K^+$ $E1B\text{-}19K^-$ (*lp5* and *dl*250), RID^- $14.7K^-$ $E1B\text{-}19K^+$ (*dl*309), RID^- $14.7K^+$ $E1B\text{-}19K^+$ (*dl*764 and *dl*7000), RID^+ $14.7K^-$ $E1B\text{-}19K^+$ (*dl*758), and RID^{++} $14.7K^{\pm}$ $E1B\text{-}19K^+$ (i.e., RID overexpressed and 14.7K underexpressed) (*pm*760). These data are consistent with those in Fig. 2 and indicate that E1B-19K and one or both of the RID and E3-14.7K proteins inhibit TRAIL-induced apoptosis of Ad-infected A549 or HeLa cells. When examined by immu-

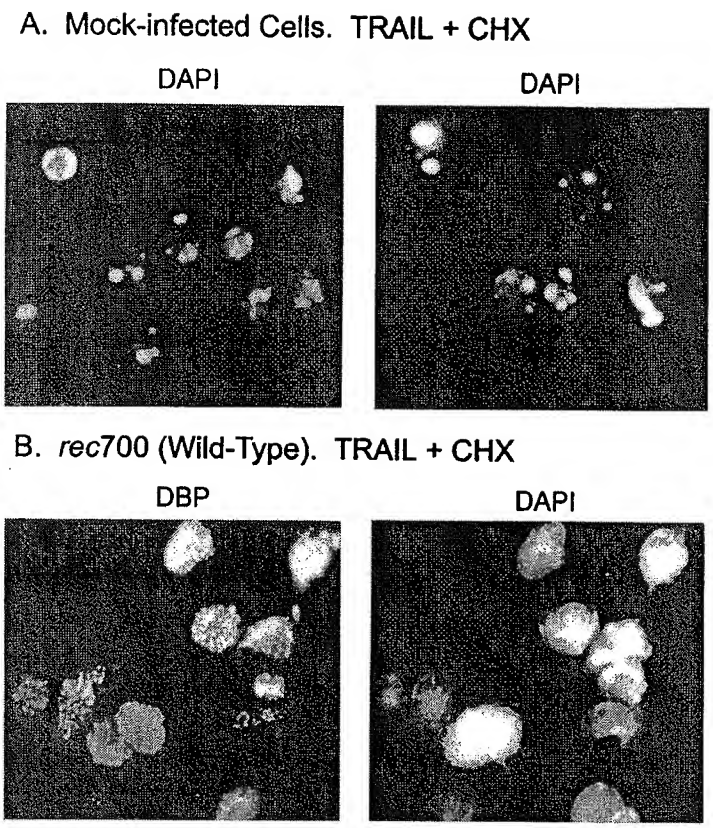

FIG. 1. Ad infection inhibits TRAIL-induced apoptosis as assessed by nuclear morphology. A549 cells were mock infected or infected with WT Ad (*rec*700) (100 PFU/cell). At 19 h p.i., the cells were treated with leucine zipper TRAIL (20 ng/ml) plus CHX (25 μg/ml). After 26 h, the cells were fixed in paraformaldehyde and then permeabilized with methanol containing DAPI. The cells were immunostained for the Ad-encoded DBP (72). (A) Two fields of mock-infected DAPI-stained nuclei. All the nuclei shown are apoptotic. (B) The same field of WT Ad-infected cells immunostained for DBP (left) and stained with DAPI (right). All the infected cells shown, as indicated by the speckled staining for DBP in the nucleus, had nonapoptotic nuclei.

nofluorescence, both mock- and *dl*111-infected cells showed loss of cytochrome *c* from mitochondria and activation of caspase 3, indicating that the cells were undergoing apoptosis (data not shown).

The mutants shown in Fig. 3A and B do not distinguish between RID and E3-14.7K because every mutant that lacks one of these proteins also expresses E1B-19K. To examine RID specifically, an experiment was conducted in human HT29.14S cells, a clone of HT29 cells selected for sensitivity to TNF, Fas agonist MAb, and lymphotoxin $\alpha_{1\text{ and }}\beta_2$ (10). In these cells, neither the E1B-19K nor E3-14.7K protein prevents Fas agonist-induced apoptosis (65), and this proves also to be true for TRAIL. TRAIL plus CHX lysed cells infected with a RID$^-$ 14.7K$^-$ E1B$^+$ mutant (*dl*309) or a RID$^-$ 14.7K$^+$ E1B$^+$ mutant (*dl*764) (Fig. 3C); since *dl*309 expresses E1B-19K and *dl*764 expresses E1B-19K and E3-14.7K, the data indicate that E3-14.7K and E1B-19K do not inhibit TRAIL-induced apoptosis in these cells. TRAIL plus CHX did not kill cells infected with three mutants that express RID, *dl*758 (14.7K$^-$), *pm*760 (RID^{++} 14.7K$^{\pm}$), and *lp*5 (E1B-19K$^-$) (Fig. 3C). These results establish that RID, but not E3-14.7K or E1B-19K, is required to inhibit TRAIL-induced apoptosis in Ad-infected HT29.14S cells.

Ad infection sensitizes cells to TRAIL-induced apoptosis, and RID is required to inhibit TRAIL-induced apoptosis in HT29.14S cells. In the results shown so far, cells were treated with TRAIL in the presence of CHX. Ad-infected HT29.14S cells were also examined for apoptosis induced by TRAIL in the absence of CHX. The cells were mock infected or infected with various mutants and treated with 0.5, 5.0, or 50 ng of TRAIL/ml at 23 h p.i., and then cell lysis was determined at 46 h p.i. by release of LDH. Mock-infected cells were not killed by TRAIL, nor were cells infected with WT Ad (*rec*700) or an E3$^+$ E1B-19K$^-$ mutant (*lp*5) (Fig. 3D). In contrast, cells were lysed by TRAIL when infected with a mutant (*dl*764) that lacks only RID and a mutant (*dl*309) that lacks RID and 14.7K (Fig. 3D).

These data support three conclusions. First, uninfected HT29.14S cells are not sensitive to TRAIL in the absence of CHX. Second, Ad infection sensitizes the cells to TRAIL; this property is revealed in the infections with the mutants that lack RID, i.e., Ad infection rendered the cells susceptible to TRAIL. Third, RID, but not E3-14.7K or E1B-19K, is required to inhibit TRAIL-induced apoptosis of Ad-infected HT29.14S cells in the absence of CHX.

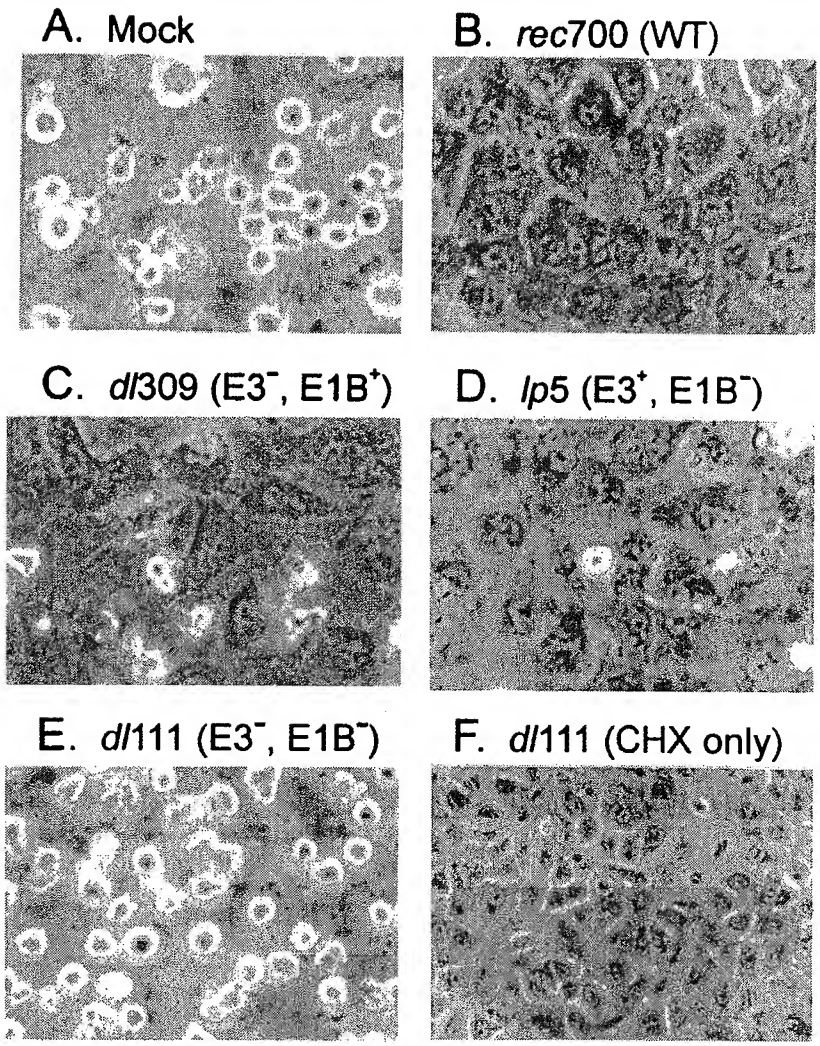

FIG. 2. Ad E3 and E1B-19K proteins inhibit TRAIL-induced apoptosis as indicated by cell morphology. A549 cells were infected with Ad mutants (100 PFU/cell), treated with leucine zipper TRAIL (200 ng/ml) plus CHX (25 μg/ml) at 14 h p.i., and then photographed after 7 h under phase-contrast microscopy. The lens magnifications were 100× (A to E) and 50× (F). See Table 1 for a description of the mutant genotypes. Mock-infected and *dl*111-infected (*dl*111 lacks E1B-19K, RID, and 14.7K) cells are apoptotic; viruses expressing E1B-19K or E3 proteins are protected. *dl*111-infected cells treated only with CHX are not apoptotic.

RID stimulates the internalization of cell surface TRAIL-R1 into putative lysosomes. We have shown that RID causes the internalization of cell surface Fas into putative endosomes, which are transported to lysosomes where Fas is degraded (72). As a result, RID inhibits Fas agonist MAb-induced apoptosis. We have also shown that RID forces the internalization of EGFR into endosomes and lysosomes, where EGFR is degraded (11, 77). We have now addressed whether this scenario also applies to TRAIL-R1. HeLa cells were mock infected, infected with WT Ad (*rec*700), or infected with a series of mutants that have individual E3 genes deleted. These mutants were *rec*700 (WT), *dl*701 (E3-6.7K$^-$), *dl*704 (E3-gp19K$^-$), *dl*731 (E3-12.5K$^-$), *pm*734.1 (ADP$^-$), *dl*762 (14.7K$^-$), and *dl*799 (RID$^-$). At 23 h p.i., unfixed cells were stained and examined by flow cytometry for cell surface TRAIL-R1 and Fas. Both receptors were removed from the cell surface by WT Ad and any mutant that expresses RID (Fig. 4). In contrast, these receptors were not cleared by the mutant (*dl*799) that lacks only RID. Similar results were obtained in A549 cells (data not shown). Thus, RID is necessary to remove TRAIL-R1 and Fas from the cell surface.

An additional point to note is that the RID$^-$ mutants express both E3-14.7K and E1B-19K. Therefore, these proteins apparently do not play a role in down-regulating these receptors.

To address whether RID causes TRAIL-R1 to internalize into endosomes and lysosomes, A549 cells were mock infected or infected with WT Ad (*rec*700) or a RID$^-$ mutant (*dl*309) and then examined for TRAIL-R1 by indirect immunofluorescence at 6 and 23 h p.i. At 6 h p.i., the Ad infection is in the

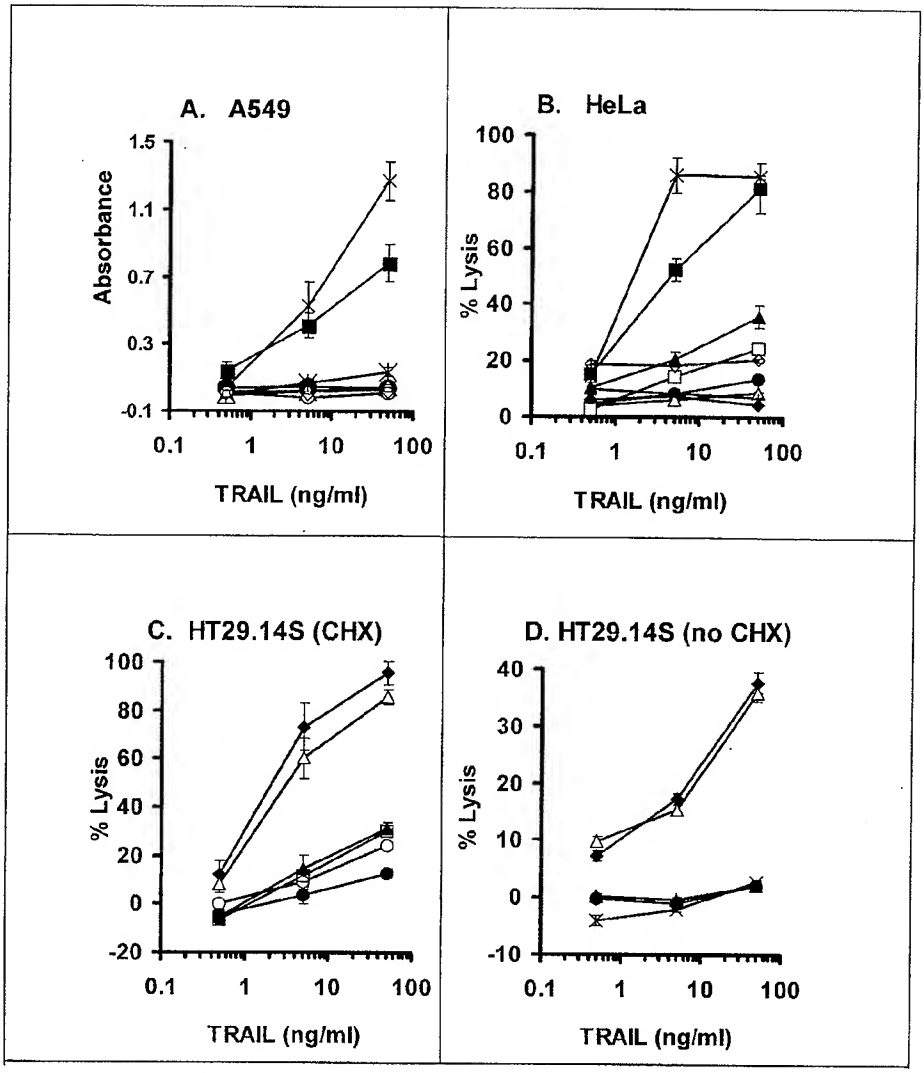

FIG. 3. The RID, E3-14.7K, and E1B-19K proteins inhibit TRAIL-induced apoptosis of Ad-infected cells. (A) Human A549 cells were infected with Ad mutants (100 PFU/cell). At 22 h p.i., the cells were treated with leucine zipper TRAIL (0.5, 5.0, and 50 ng/ml) plus CHX (25 μg/ml). After 28 h, apoptosis was determined colorimetrically based on release of LDH. The viruses used were mock, *rec*700, *dl*111, *dl*309, *lp*5, *dl*250, *pm*760, *dl*758, *dl*764, and *dl*7000. (B) Human HeLa cells infected with Ad mutants (100 PFU/cell) were treated with TRAIL and CHX at 21 h p.i. and assayed for LDH release after 24 h of treatment. The viruses used were mock, *rec*700, *dl*111, *dl*309, *lp*5, *dl*764, *dl*758, *dl*7000, and Ad5. (C) Human HT29.14S cells were infected with Ad mutants (100 PFU/cell). At 22 h p.i., the cells were treated with TRAIL and CHX. An LDH assay was done after 26 h of treatment. The viruses used were *rec*700, *dl*309, *lp*5, *dl*764, *dl*758, and *pm*760. (D) Human HT29.14S cells were infected with Ad mutants (100 PFU/cell). The cells were treated with TRAIL (no CHX) at 23 h p.i. and assayed for LDH release after 23 h of TRAIL treatment. The viruses used were mock, *rec*700, *dl*309, *dl*764, and *lp*5. ×, mock; ■, *dl*111 (RID$^-$ 14.7K$^-$ E1B$^-$); ●, *rec*700 (WT); ◆, *dl*309 (RID$^-$ 14.7K$^-$); ▲, *lp*5 (E1B$^-$); *, *dl*250 (E1B$^-$); △, *dl*764 (RIDβ$^-$); □, *dl*758 (14.7K$^-$); ○, *pm*760 (RID$^+$ increased; 14.7K$^+$ decreased); ◇, *dl*7000 (RID$^-$); +, Ad5 (WT). The error bars indicate standard deviations.

early phase (prior to Ad DNA replication), when RID is present in quite small amounts. At 23 h, the infection is in late stages, and RID has been in the cell for about 20 h and has accumulated in larger amounts. With mock-infected cells, there was uniform TRAIL-R1 staining on the cell surface and also some internal Golgi-like punctate staining (Fig. 5A and D). With WT Ad at 6 h, TRAIL-R1 had been cleared from the surfaces of about half the cells, and many vesicles were apparent (Fig. 5B). At 23 h, there was very little staining of TRAIL-R1, and only a few cells had vesicles (the field shown in Fig. 5E was selected to show these few cells). With the RID$^-$ mutant (*dl*309), TRAIL-R1 was not removed from the cell surface (Fig. 5C and F). Thus, RID causes internalization of cell surface TRAIL-R1 into putative endosomes and lysosomes, resulting in disappearance, presumably degradation, of TRAIL-R1. Attempts to show by immunoblotting that RID causes degradation of TRAIL-R1 were unsuccessful because TRAIL-R1 could not be detected.

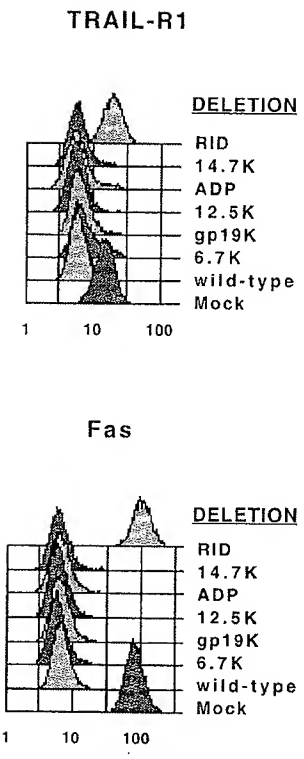

FIG. 4. RID is required for internalization of TRAIL-R1 and Fas from the surface of Ad-infected HeLa cells. Cells were mock-infected or infected (150 PFU/cell) with WT Ad (*rec*700) or with E3 deletion mutants in which the genes for the individual E3 proteins have been deleted. At 23 h p.i., unfixed cells were stained with MAbs against TRAIL-R1 or Fas, incubated with goat anti-mouse FITC-conjugated secondary antibody, and analyzed by flow cytometry using a FACScaliber flow cytometer and Cell Quest software (72). The mutants are as follows: *rec*700 (WT), *dl*701 (E3-6.7K$^-$), *dl*704 (E3-gp19K$^-$), *dl*731 (E3-12.5K$^-$), *pm*734.1 (ADP$^-$), *dl*762 (E3-14.7K$^-$), and *dl*799 (RID$^-$).

If TRAIL-R1 is degraded in lysosomes, then its degradation should be inhibited by Baf. Baf, a specific inhibitor of the vacuolar-type H^+ ATPase, prevents acidification of vesicles and the sorting of receptors from late endosomes to lysosomes (79, 91). Baf inhibits the RID-mediated degradation of Fas (72) and EGFR (data not shown). In the present study, Baf had only a marginal effect on mock- or *dl*309-infected A549 cells, causing a modest accumulation of TRAIL-R1-containing vesicles, probably by blocking a low level of constitutive degradation of TRAIL-R1 (Fig. 5G and I). With WT Ad infection (*rec*700), Baf caused TRAIL-R1 to accumulate in large vesicles rather than being degraded (Fig. 5H). Baf did not affect clearance of TRAIL-R1 from the cell surface. These results strongly support the conclusion that RID mediates the degradation of TRAIL-R1 in lysosomes.

RID and E3-14.7K are sufficient to inhibit TRAIL-induced apoptosis. The results shown above address, in the context of Ad infection, whether RID, E3-14.7K, and E1B-19K are required to inhibit TRAIL-induced apoptosis and force TRAIL-R1 from the cell surface into putative endosomes and lysosomes. As a means to examine whether the E3 proteins are sufficient for these TRAIL-related effects, we employed replication-defective Ad vectors that express E3 proteins (K. Toth, M. Kuppuswamy, K. Doronin, O. A. Doronina, A. E. Tollefson, and W. S. M. Wold, unpublished data). One vector, named Ad/E3, contains an expression cassette with the entire E3 transcription unit driven by the CMV promoter-enhancer. Six E3 proteins are expressed from this vector, namely, RIDα, RIDβ, E3-14.7K, E3-gp19K, E3-6.7K, and E3-12.5K (data not shown). Other Ad proteins are not synthesized. A second vector, named Ad/RID/14.7K, expresses only RID and E3-14.7K from the CMV promoter. A third vector, named Ad/RID, expresses only RID from the CMV promoter. A fourth vector, named Ad/14.7K, expresses only E3-14.7K from the CMV promoter. The control for the Ad vectors was infection with an empty Ad vector (Ad/null). A549 cells were mock infected or infected with WT Ad (*rec*700) or the Ad vectors. The cells were treated with TRAIL plus CHX at 24.5 h p.i., and apoptosis was measured at 52.5 h p.i. by release of LDH. Mock-infected cells were lysed efficiently by TRAIL, and this lysis was strongly inhibited by WT Ad (Fig. 6A). The RID-expressing vectors also provided strong protection against TRAIL; the protection was slightly less than that of WT Ad, probably because the vectors lack E1B-19K (Fig 6A). Ad/14.7K gave partial but significant protection, especially when TRAIL was added at 5 ng/ml (Fig. 6A). These results show that RID and, to a lesser extent, E3-14.7K are sufficient, in the context of these Ad vectors, to inhibit TRAIL-induced apoptosis. The RID-expressing vectors also efficiently inhibited apoptosis mediated through the Fas pathway (data not shown).

To address further whether E3-14.7K can function alone to block TRAIL-induced apoptosis, two clonal lines of stably transfected A549 cells expressing E3-14.7K were examined. These lines express good levels of E3-14.7K that are readily detected by immunoblotting or immunofluorescence (data not shown). Apoptosis was determined by a trypan blue exclusion assay. Most parental A549 cells were killed by TRAIL plus CHX, whereas about 60% of both cell lines remained viable (Fig. 6B). A number of additional E3-14.7K-expressing clones had similar phenotypes (data not shown). Thus, E3-14.7K expression is sufficient to inhibit TRAIL-induced apoptosis in these cell lines.

The Ad vectors were also used to address whether RID is sufficient to clear TRAIL-R1 from the cell surface. HeLa cells were mock infected, infected with *rec*700, or infected with the Ad vectors. At 23 h p.i., the cells were stained for TRAIL-R1 or TfnR and then analyzed by flow cytometry. TfnR, the negative control, was not affected (Fig. 7). TRAIL-R1 was cleared by *rec*700 and all vectors that express RID (Fig. 7). TRAIL-R1 was not cleared by Ad/14.7K or the empty vector.

The Ad vectors were also used to examine the internalization of TRAIL-R1 into vesicles in the absence and presence of Baf. A549 cells were mock-infected or infected with the vectors, treated with Baf (0.1 μM) at 3 h p.i., and then fixed and stained for TRAIL-R1 at 25 h p.i. With mock-infected cells not treated with Baf, there was strong staining for TRAIL-R1 on the cell surface (Fig. 8). Baf had little effect on mock-infected cells. With the Ad/E3, Ad/RID/14.7K, and Ad/RID infections at 25 h p.i., most cells were no longer stained for TRAIL-R1, and with those that did stain, TRAIL-R1 was in vesicles rather than on the cell surface (Fig. 8). Similar results were obtained

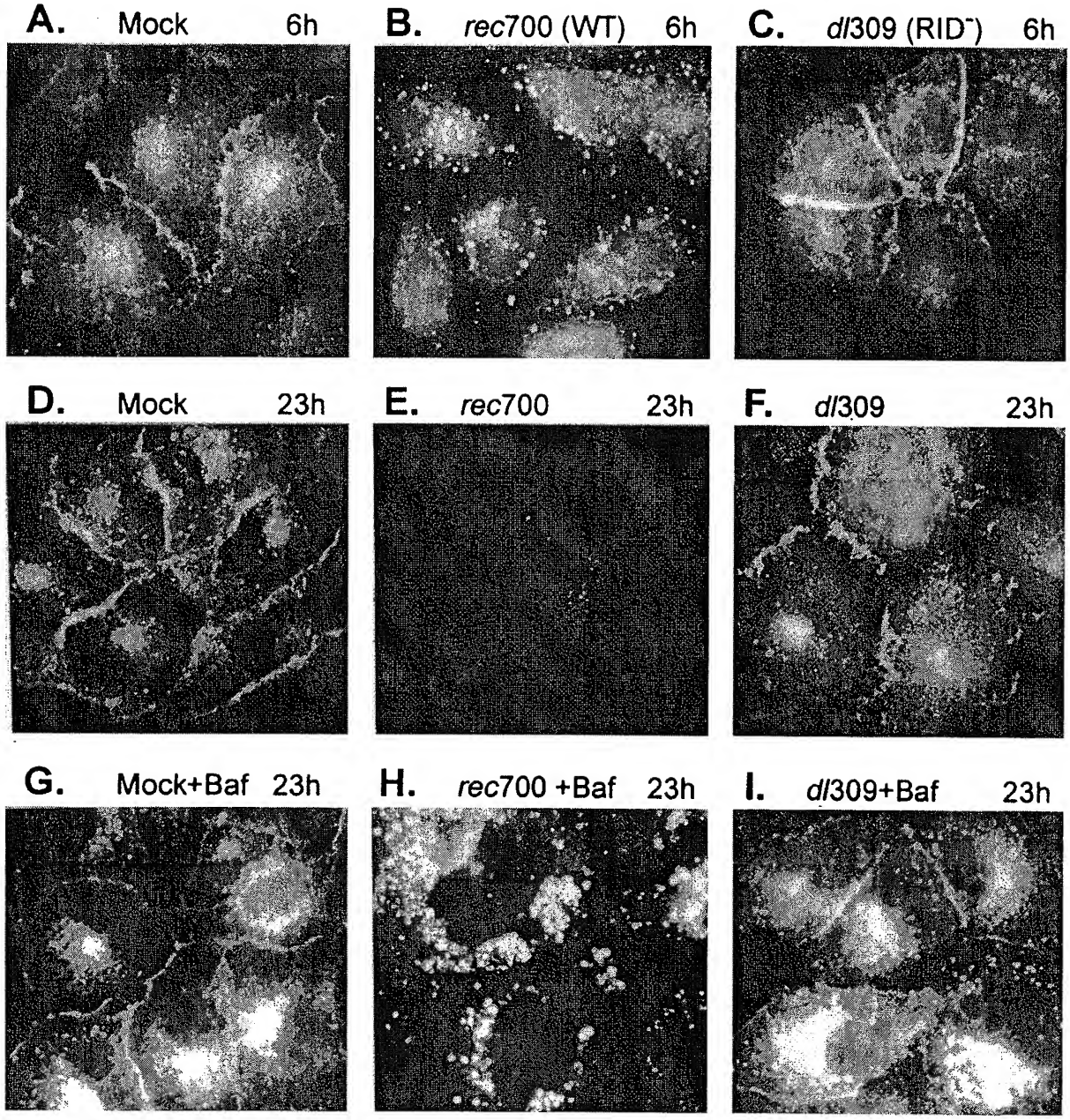

FIG. 5. RID mediates internalization of cell surface TRAIL-R1 into putative endosomes and lysosomes, where TRAIL-R1 is degraded. (A to F) Cells were mock infected or infected with WT Ad (*rec*700) or a RID$^-$ 14.7K$^-$ mutant (*dl*309). At 6 and 23 h p.i., the cells were fixed in paraformaldehyde and then permeabilized with methanol. The cells were immunostained for TRAIL-R1 using the M271 MAb (Immunex) and FITC-conjugated goat anti-mouse IgG. For the 6- and 23-h time points, 400 and 50 PFU of virus/cell, respectively, were used. (G to I) Same as for panels D to F, except the cells were treated with Baf (0.1 μM) beginning at 6 h p.i.

in a parallel experiment in which the cells were immunostained for EGFR (data not shown). When cells infected with RID-expressing vectors were treated with Baf, TRAIL-R1 was cleared from the cell surface and it accumulated in brightly staining vesicles (Fig. 8). These results are similar to those observed with *rec*700 (Fig. 5). The Ad/14.7K vector, which express E3-14.7K but not RID, did not affect TRAIL-R1 localization (data not shown). We conclude that RID expressed from the vectors is sufficient to force TRAIL-R1 from the cell surface into vesicles and to cause degradation (disappearance) of TRAIL-R1. The degradation of TRAIL-R1 very likely occurs in lysosomes, because it was inhibited by Baf.

DISCUSSION

We have shown that Ad has three independent proteins that inhibit TRAIL-induced apoptosis, RID, E3-14.7K, and E1B-

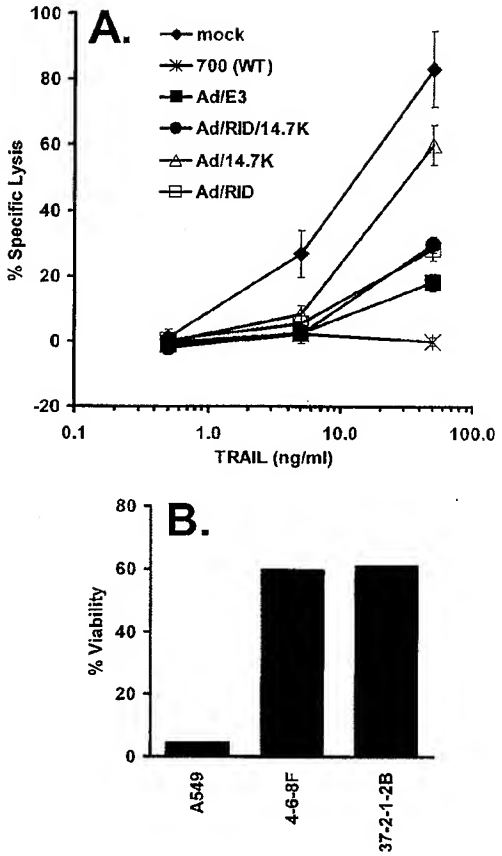

FIG. 6. RID protein is sufficient to inhibit TRAIL-induced apoptosis; 14.7K protects cells from TRAIL-induced apoptosis in stable transfectants. (A) A549 cells were mock infected or infected with 5 to 20 PFU of WT Ad (*rec*700), Ad/E3 (expressing all E3 proteins), Ad/RID/14.7K, Ad/RID, or Ad/14.7K per cell and then treated with TRAIL (plus CHX at 25 μg/ml) at 24.5 h p.i. as indicated. After 28 h, apoptosis was determined by release of LDH. The error bars indicate standard deviations. (B) A549 cells as well as two independent stably transfected A549 clonal cell lines expressing the E3-14.7K protein, named 4-6-8F (WT 14.7K expression) and 37-2-1-2B (14.7K with point mutation of D37A, not affecting function), were treated with TRAIL (1.0 ng/ml) plus CHX (25 μg/ml). After 25 h of treatment, the percentage of viable cells was determined by trypan blue exclusion.

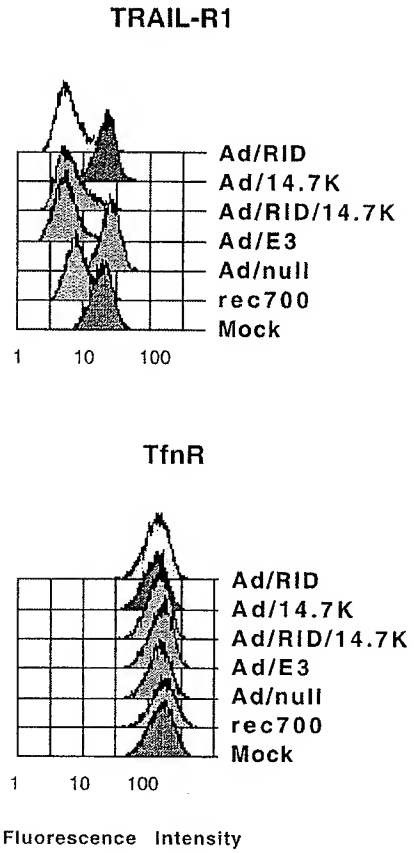

FIG. 7. Infection with replication-defective E1-minus Ad vectors expressing RID removes TRAIL-R1 but not TfnR from the cell surface. HeLa cells were mock infected or infected with 5 to 20 PFU of *rec*700 or Ad vectors/cell. At 23 h p.i., unfixed cells were stained with MAbs for TRAIL-R1 or TfnR and assayed by flow cytometry. Ad/E3 encodes and expresses all E3 proteins except ADP (E3-12.5K, E3-6.7K, E3-gp19K, RIDα, RIDβ, and E3-14.7K). Ad/E3, Ad/RID/14.7K, Ad/RID, and Ad/14.7K express only the E3 proteins indicated.

19K. RID stimulates the internalization of TRAIL-R1 from the cell surface, which probably explains why RID inhibits killing by TRAIL. TRAIL-R1 is internalized into vesicles, putative endosomes, and lysosomes and is degraded as indicated by the severe reduction in immunostaining for TRAIL-R1. (An alternative explanation for the lack of TRAIL-R1 immunostaining is that the epitope for TRAIL-R1 becomes masked.) The inhibition of TRAIL-R1 degradation by Baf argues strongly that TRAIL-R1 is degraded in lysosomes.

There are six known members of the TNFR superfamily that have death domains, namely, TNFR1, Fas, death receptor 3, TRAIL-R1, TRAIL-R2, and death receptor 6. Ligand-induced activation of TNFR1, Fas, TRAIL-R1, and TRAIL-R2 causes a series of protein-protein interactions involving the death domains that leads to activation of caspases and apoptosis (reviewed in references 1, 2, 17, 53, and 82). RID, E1B-19K, and E3-14.7K inhibit apoptosis induced by three of these ligands, TNF, FasL, and TRAIL (references 19, 21, 22, 24, 65, and 72 and this study). The Ad proteins block death ligand-induced apoptosis at several levels. RID gets rid of TRAIL-R1 and Fas by forcing them from the cell surface into lysosomes, where they are degraded. While this report was in preparation, Benedict et al. (4) reported that in addition to RID (referred to as E3-10.4K/14.5K), an E3 protein named E3-6.7K is required to clear TRAIL from the cell surface. Their studies were conducted with retroviruses expressing the RID and/or E3-6.7K proteins. We clearly have not observed a requirement for E3-6.7K in RID-mediated down-regulation of TRAIL-R1. Perhaps these differences in results are due to different experimental systems. Benedict et al. (4) also reported that both RID and E3-6.7K are required to down-regulate TRAIL-R2. We have similar findings under our experimental conditions (A. E. Tollefson, D. L. Lichtenstein, M. Kuppuswamy, K. Toth, K. Doronin, O. A. Doronina, C. A. Smith, and W. S. M. Wold, unpublished data).

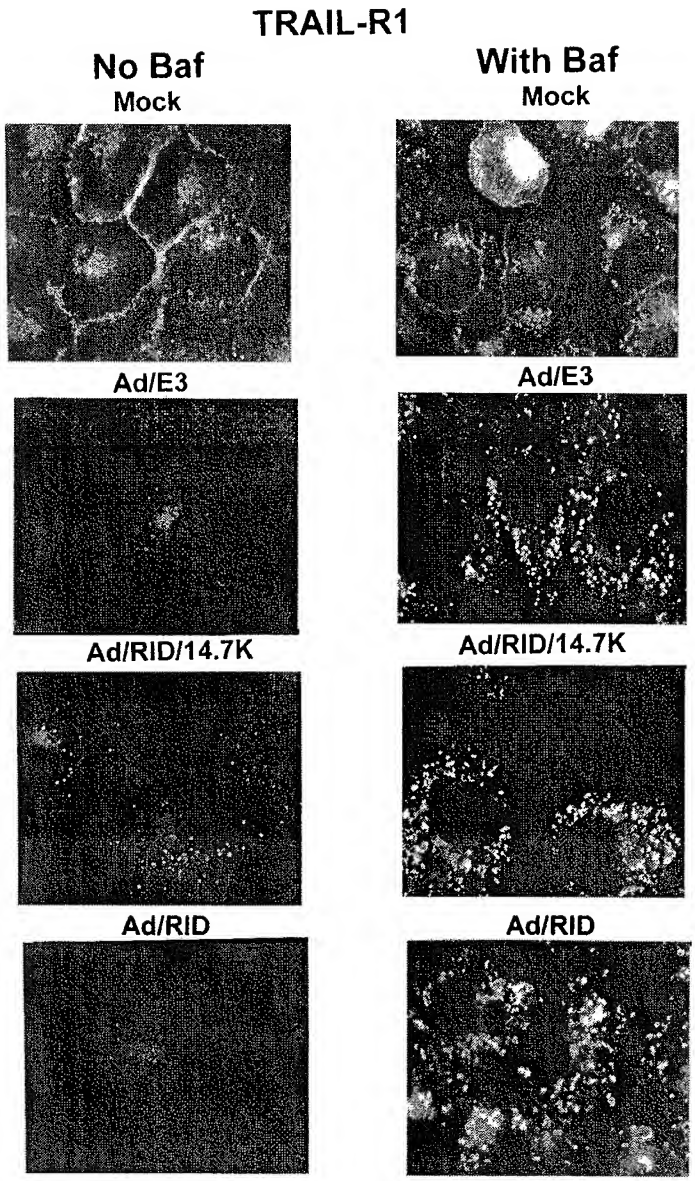

FIG. 8. Expression of RID by Ad vectors mediates down-regulation and degradation of TRAIL-R1, presumably by a lysosomal degradation pathway. A549 cells were mock infected or infected with 50 to 200 PFU of Ad vectors/cell. Baf was added at 3 h p.i. The cells were fixed and immunostained for TRAIL-R1 at 25 h p.i.

RID also inhibits TNF-induced translocation of the 85-kDa cytosolic phospholipase A_2 ($cPLA_2$) from the cytosol to membranes (18). There is evidence that TNF-induced activation of $cPLA_2$ is important in TNF-induced apoptosis (32, 42, 71, 86). We do not know if $cPLA_2$ is activated through the Fas and TRAIL pathways.

E3-14.7K apparently inhibits apoptosis at more than one level. Yeast two-hybrid studies indicate that E3-14.7K binds to three cellular proteins named FIP-1, FIP-2, and FIP-3 (45–47). FIP-2 and FIP-3 may be part of the apoptotic signaling pathway that leads from TNFR1 and Fas through RIP; E3-14.7K could potentially inhibit this pathway. E3-14.7K is also reported to inhibit apoptosis induced by an Ad vector expressing FasL or by transient transfection of procaspase 8 (13). E3-14.7K forms a complex with procaspase 8 (13). E3-14.7K also inhibits TNF-induced activation of $cPLA_2$ (42, 71, 92). It is not known if any of these functions of E3-14.7K account for its ability to inhibit TRAIL-induced apoptosis.

E1B-19K is a functional homolog of BCL-2 (7). It interacts with and inhibits the activity of proapoptotic members of the BCL-2 family (14, 83, 87). These proteins appear to promote apoptosis in part by displacing adapters from antiapoptotic BCL-2 family members, allowing caspases to become activated. E1B-19K was recently reported to interact with a con-

formationally altered form of Bax in mitochondria; this interaction inhibits cytochrome *c* release and caspase-9 activation (57). E1B-19K did not prevent activation of caspase-8. It seems likely that these functions of E1B-19K explain why E1B-19K inhibits TRAIL-induced apoptosis.

Routes et al. (60) recently reported that the Ad E1A proteins sensitize human A2058 melanoma and H4 fibrosarcoma cells to TRAIL-dependent killing. Such a function of E1A would explain the results of our experiment (Fig. 3D) showing that Ad infection sensitizes HT29.14S cells to TRAIL-induced apoptosis. Routes et al. (60) also reported an experiment suggesting that unspecified E3 proteins, and to a lesser extent E1B-19K, are required to inhibit TRAIL killing of Ad-infected A2058 cells.

Apoptosis would be deleterious to virus replication if it should occur before replication is complete (15). Thus, it makes sense that the virus should target receptors that mediate apoptosis. Apoptosis is a major mechanism by which the immune system eliminates unwanted cells (53). CTL kill through two main systems, the perforin-granzymes and Fas. They also kill through the TNF pathway, as indicated by long-term cell culture cytolysis assays. It is likely that the TRAIL system is involved in CTL killing of virus-infected cells, considering that T cells express TRAIL. Ad is well equipped to prevent killing of infected cells by CTL. The Ad-encoded gp19K prevents recognition of infected cells by the T-cell receptor. The RID, E3-14.7K, and E1B-19K proteins block apoptosis induced through Fas, TNFR1, and TRAIL-R1. Activated NK cells kill through the perforin-granzyme and perhaps the Fas and TRAIL systems, and activated macrophages synthesize TNF. Thus, it is possible that these Ad proteins inhibit CTL killing not only at the adaptive stage of the immune response but also at the innate stage.

Other signal transduction pathways, including NF-κB, stress-induced kinases, and neutral and acidic sphingomyelinases, are also activated through the TNFR1, Fas, and TRAIL receptors (17, 25, 53). Perhaps activation of one or more of these signaling pathways is deleterious to Ad replication, and accordingly, the receptors are eliminated by RID. The idea would be consistent with the ability of RID to cause internalization and degradation of EGFR, insulin receptor, and insulinlike growth factor-1 (IGF-1) receptor (11, 43, 77). For example, $cPLA_2$ can be activated not only by TNF, but also by growth factors through the Ras–mitogen-activated protein kinase pathway. Arachidonic acid is the precursor to the proinflammatory eicosinoids, and by inhibiting arachidonic acid synthesis, the Ad proteins could inhibit inflammation. Indeed, the RID and E3-14.7K proteins inhibit inflammation in mouse models (29, 30, 66).

Considering that RID down-regulates very distinct receptors in the TNFR and protein tyrosine kinase families, the question arises as to the specificity of RID. There are some receptors that are not affected by RID, namely, TfnR (Fig. 7) (72, 77), major histocompatibility complex class I antigens (33), platelet-derived growth factor receptor, HER2 (43), CD46 (19), and CD44 (unpublished results). Thus, there is considerable specificity to RID.

As discussed above, RID causes the receptors for TRAIL, FasL, TNF, EGF, insulin, and IGF-1 to be internalized from the cell surface and degraded in lysosomes (11, 43, 72, 77; T. Dimitrov, C. F. Colle, A. E. Tollefson, D. L. Lichtenstein, and W. S. M. Wold, unpublished data). EGF, insulin, and IGF-1 are well known to stimulate internalization and degradation of their receptors. Surprisingly, little is known about this property for the death receptor ligands. Growth factor-induced degradation of receptors is believed to attenuate the growth factor signal, and this could also be true for the death receptor ligands. Attenuation may not be necessary if the cell has already been triggered to die. However, many cells are normally resistant to these death ligands and must become sensitized in order for the cell to undergo apoptosis; for nonsensitized cells, ligand-induced clearance of the death receptors would preclude cell death if the cells should subsequently receive a sensitizing signal. In any case, RID appears to serve as a surrogate ligand to cause internalization and degradation of these receptors. (There is no evidence that RID performs the other response to these ligands, i.e., induction of signal transduction.) The molecular mechanism of action of RID must be very interesting, and knowledge of it will increase our understanding of receptor signaling and sorting.

ACKNOWLEDGMENTS

K.T., K.D., and M.K. contributed equally to this work.

We thank Chris Wells for technical assistance, Todd Ranheim for *dl*7000, Abraham Scaria for *pm*734.1, G. Chinnadurai for *lp*5 and *dl*250, Tom Shenk for *dl*309, Harold Ginsberg and Lee Babiss for *dl*111, Maurice Green for antiserum to DBP, Jeff Browning for HT29.14S cells, Lynn Dustin for assistance with flow cytometry, Jayma Mikes for preparation of the figures, and Dawn Schwartz for preparation of the manuscript.

This work was supported by grants CA58538 and CA24710 from the National Institutes of Health.

REFERENCES

1. **Ashkenazi, A., and V. M. Dixit** 1999. Apoptosis control by death and decoy receptors. Curr. Opin. Cell. Biol. **11**:255–260.
2. **Ashkenazi, A., and V. M. Dixit.** 1998. Death receptors: signaling and modulation. Science **281**:1305–1308.
3. **Babiss, L. E., P. B. Fisher, and H. S. Ginsberg.** 1984. Effect on transformation of mutations in the early region 1b-encoded 21- and 55-kilodalton proteins of adenovirus 5. J. Virol. **52**:389–395.
4. **Benedict, C., P. Norris, T. Prigozy, J. L. Bodmer, J. A. Mahr, C. Garnett, F. Martinon, J. Tschopp, L. R. Gooding, and C. F. Ware.** 2001. Three adenovirus E3 proteins cooperate to evade apoptosis by tumor necrosis factor-related apoptosis-inducing ligand receptor-1 and -2. J. Biol. Chem. **276**: 3270–3278.
5. **Bett, A. J., W. Haddara, L. Prevec, and F. L. Graham.** 1994. An efficient and flexible system for construction of adenovirus vectors with insertions or deletions in early regions 1 and 3. Proc. Natl. Acad. Sci. USA **91**:8802–8806.
6. **Bhat, B. M., and W. S. M. Wold.** 1987. A small deletion distant from a splice or polyadenylation site dramatically alters pre-mRNA processing in region E3 of adenovirus. J. Virol. **61**:3938–3945.
7. **Boyd, J. M., S. Malstrom, T. Subramanian, L. K. Venkatesh, U. Schaeper, B. Elangovan, C. D'Sa-Eipper, and G. Chinnadurai.** 1994. Adenovirus E1B 19 kDa and Bcl-2 proteins interact with a common set of cellular proteins. Cell **79**:341–351.
8. **Brady, H. A., A. Scaria, and W. S. M. Wold.** 1992. Map of *cis*-acting sequences that determine alternative pre-mRNA processing in the E3 complex transcription unit of adenovirus. J. Virol. **66**:5914–5923.
9. **Brady, H. A., and W. S. M. Wold.** 1988. Competition between splicing and polyadenylation reactions determines which adenovirus region E3 mRNAs are synthesized. Mol. Cell. Biol. **8**:3291–3297.
10. **Browning, J. L., K. Miatkowski, I. Sizing, D. Griffiths, M. Zafari, C. D. Benjamin, W. Meier, and F. Mackay.** 1996. Signaling through the lymphotoxin beta receptor induces the death of some adenocarcinoma tumor lines. J. Exp. Med. **183**:867–878.
11. **Carlin, C. R., A. E. Tollefson, H. A. Brady, B. L. Hoffman, and W. S. M. Wold.** 1989. Epidermal growth factor receptor is down-regulated by a 10,400 MW protein encoded by the E3 region of adenovirus. Cell **57**:135–144.
12. **Chaudhary, P. M., M. Eby, A. Jasmin, A. Bookwalter, J. Murray, and L. Hood.** 1997. Death receptor 5, a new member of the TNFR family, and DR4 induce FADD-dependent apoptosis and activate the NF-kB pathway. Immunity **7**:821–830.

13. **Chen, P., J. Tian, I. Kovesdi, and J. B. Bruder.** 1998. Interaction of the adenovirus 14.7K protein with FLICE inhibits Fas ligand-induced apoptosis. J. Biol. Chem. **273**:5815–5820.
14. **Chinnadurai, G.** 1998. Control of apoptosis by human adenovirus genes. Semin. Virol. **8**:399–408.
15. **Chiou, S. K., and E. White.** 1998. Inhibition of ICE-like proteases inhibits apoptosis and increases virus production during adenovirus infection. Virology **244**:108–118.
16. **Clarke, P., S. Meintzer, S. Gibson, C. Widmann, T. Garrington, G. Johnson, and K. Tyler.** 2000. Reovirus-induced apoptosis is mediated by TRAIL. J. Virol. **74**:8135–8139.
17. **Darnay, B. G., and B. B. Aggarwal.** 1997. Early events in TNF signaling: a story of associations and dissociations. J. Leukoc. Biol. **61**:559–566.
18. **Dimitrov, T., P. Krajcsi, T. W. Hermiston, A. E. Tollefson, M. Hannink, and W. S. M. Wold.** 1997. Adenovirus E3-10.4K/14.5K protein complex inhibits tumor necrosis factor-induced translocation of cytosolic phospholipase A_2 to membranes. J. Virol. **71**:2830–2837.
19. **Elsing, A. and H.-G. Burgert.** 1998. The adenovirus E3/10.4K-14.5K proteins down-modulate the apoptosis receptor Fas/Apo-1 by inducing its internalization. Proc. Natl. Acad. Sci. USA **95**:10072–10077.
20. **Fanger, N., C. Maliszewski, K. Schooley, and T. S. Griffith.** 1999. Human dendritic cells mediate cellular apoptosis via tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). J. Exp. Med. **190**:1155–1164.
21. **Gooding, L. R., L. Aquino, P. J. Duerksen-Hughes, D. Day, T. M. Horton, S. P. Yei, and W. S. M. Wold.** 1991. The E1B 19,000-molecular-weight protein of group C adenoviruses prevents tumor necrosis factor cytolysis of human cells but not of mouse cells. J. Virol. **65**:3083–3094.
22. **Gooding, L. R., L. W. Elmore, A. E. Tollefson, H. A. Brady, and W. S. M. Wold.** 1988. A 14,700 MW protein from the E3 region of adenovirus inhibits cytolysis by tumor necrosis factor. Cell **53**:341–346.
23. **Gooding, L. R., T. S. Ranheim, A. E. Tollefson, L. Aquino, P. Duerksen-Hughes, T. M. Horton, and W. S. M. Wold.** 1991. The 10,400- and 14,500-dalton proteins encoded by region E3 of adenovirus function together to protect many but not all mouse cell lines against lysis by tumor necrosis factor. J. Virol. **65**:4114–4123.
24. **Gooding, L. R., I. O. Sofola, A. E. Tollefson, P. Duerksen-Hughes, and W. S. M. Wold.** 1990. The adenovirus E3-14.7K protein is a general inhibitor of tumor necrosis factor-mediated cytolysis. J. Immunol. **145**:3080–3086.
25. **Griffith, T., and D. H. Lynch.** 1998. TRAIL: a molecule with multiple receptors and control mechanisms. Curr. Opin. Immunol. **10**:559–563.
26. **Griffith, T. S., C. T. Rauch, P. J. Smolak, J. Y. Waugh, N. Boiani, D. H. Lynch, C. A. Smith, R. G. Goodwin, and M. Z. Kubin.** 1999. Functional analysis of TRAIL receptors using monoclonal antibodies. J. Immunol. **162**:2597–2605.
27. **Griffith, T. S., S. R. Wiley, M. Kubin, L. Sedger, C. Maliszewski, and N. Fanger.** 1999. Monocyte-mediated tumoricidal activity via the tumor necrosis factor-related cytokine, TRAIL. J. Exp. Med. **189**:1343–1353.
28. **Gross, A., J. McDonnell, and S. Korsmeyer.** 1999. BCL-2 family members and the mitochondria in apoptosis. Genes Dev. **13**:1899–1911.
29. **Harrod, K. S., T. W. Hermiston, B. C. Trapnell, W. S. M. Wold, and J. A. Whitsett.** 1998. Lung-specific expression of E3-14.7K in transgenic mice attenuates adenoviral vector-mediated lung inflammation and enhances transgene expression. Hum. Gene Ther. **9**:1885–1898.
30. **Harrod, K. S., and A. D. W. J. A. Mounday.** 2000. Adenoviral E3-14.7K protein in LPS-induced lung inflammation. Am. J. Physiol. Lung Cell Mol. Physiol. **278**:631–639.
31. **Hashimoto, S., A. Ishii, and S. Yonehara.** 1991. The E1b oncogene of adenovirus confers cellular resistance to cytotoxicity of tumor necrosis factor and monoclonal anti-Fas antibody. Int. Immunol. **3**:343–351.
32. **Hayakawa, M., N. Ishida, K. Takeuchi, S. Shibamoto, T. Hori, N. Oku, F. Ito, and M. Tsujimoto.** 1993. Arachidonic acid-selective cytosolic phospholipase A_2 is crucial in the cytotoxic action of tumor necrosis factor. J. Biol. Chem. **268**:11290–11295.
33. **Hermiston, T. W., R. A. Tripp, T. Sparer, L. R. Gooding, and W. S. M. Wold.** 1993. Deletion mutation analysis of the adenovirus type 2 E3-gp19K protein: identification of sequences within the endoplasmic reticulum lumenal domain that are required for class I antigen binding and protection from adenovirus-specific cytotoxic T lymphocytes. J. Virol. **67**:5289–5298.
34. **Hoffman, P., M. B. Yaffe, B. L. Hoffman, S. Yei, W. S. M. Wold, and C. Carlin.** 1992. Characterization of the adenovirus E3 protein that down-regulates the epidermal growth factor receptor. Evidence for intermolecular disulfide bonding and plasma membrane localization. J. Biol. Chem. **267**:13480–13487.
35. **Jeremias, I., I. Herr, T. Boehler, and K.-M. Debatin.** 1998. TRAIL/Apo-2-ligand-induced apoptosis in human T cells. Eur. J. Immunol. **28**:143–152.
36. **Jones, N., and T. Shenk.** 1979. Isolation of adenovirus type 5 host range deletion mutants defective for transformation of rat embryo cells. Cell **17**:683–689.
37. **Kashii, Y., R. Giorda, R. Herberman, T. Whiteside, and N. Vujanovic.** 1999. Constitutive expression and role of the TNF family ligands in apoptotic killing of tumor cells by human NK cells. J. Immunol. **163**:5358–5366.
38. **Katsikis, P. D., M. E. Garcia-Ojeda, J. F. Torres-Roca, I. M. Tijoe, C. A. Smith, L. A. Herzenberg, and L. A. Herzenberg.** 1997. Interleukin-1 beta converting enzyme-like protease involvement in Fas-induced and activation-induced peripheral blood T cell apoptosis in HIV infection. TNF-related apoptosis-inducing ligand can mediate activation-induced T cell death in HIV infection. J. Exp. Med. **186**:1365–1372.
39. **Kayagaki, N., N. Yamaguchi, M. Nakayama, A. Kawasaki, H. Akiba, K. Okumura, and H. Yagita.** 1999. Involvement of TNF-related apoptosis-inducing ligand in human $CD4^+$ T cell-mediated cytotoxicity. J. Immunol. **162**:2639–2647.
40. **Kayakaki, N., N. Yamaguchi, M. Nakayama, H. Eto, K. Okumura, and H. Yagita.** 1999. Type I interferons (IFNs) regulate tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) expression on human T cells: a novel mechanism for the antitumor effects of type I IFNs. J. Exp. Med. **189**:1451–1460.
41. **Kirstein, M., and C. Baglioni.** 1986. Tumor necrosis factor induces synthesis of two proteins in human fibroblasts. J. Biol. Chem. **261**:9565–9567.
42. **Krajcsi, P., T. Dimitrov, T. W. Hermiston, A. E. Tollefson, T. S. Ranheim, S. B. Vande Pol, A. H. Stephenson, and W. S. M. Wold.** 1996. The adenovirus E3-14.7K protein and the E3-10.4K/14.5K complex of proteins, which independently inhibit tumor necrosis factor (TNF)-induced apoptosis, also independently inhibit TNF-induced release of arachidonic acid. J. Virol. **70**:4904–4913.
43. **Kuivinen, E., B. L. Hoffman, P. A. Hoffman, and C. R. Carlin.** 1993. Structurally related class I and class II receptor protein tyrosine kinases are down-regulated by the same E3 protein coded for by human group C adenoviruses. J. Cell Biol. **120**:1271–1279.
44. **Leverkus, M., M. Neumann, T. Mengling, C. Rauch, E.-B. Brocker, P. H. Krammer, and H. Walczak.** 2000. Regulation of tumor necrosis factor-related apoptosis-inducing ligand sensitivity in primary and transformed human keratinocytes. Cancer Res. **60**:553–559.
45. **Li, Y., J. Kang, J. Friedman, L. Tarassishin, J. Ye, A. Kovalenko, D. Wallach, and M. S. Horwitz.** 1999. Identification of a cell protein (FIP-3) as a modulator of NF-κB activity and as a target of an adenovirus inhibitor of tumor necrosis factor α-induced apoptosis. Proc. Natl. Acad. Sci. USA **96**:1042–1047.
46. **Li, Y., J. Kang, and M. S. Horwitz.** 1997. Interaction of an adenovirus 14.7-kilodalton protein inhibitor of tumor necrosis factor alpha cytolysis with a new member of the GTPase superfamily of signal transducers. J. Virol. **71**:1576–1582.
47. **Li, Y., J. Kang, and M. S. Horwitz.** 1998. Interaction of an adenovirus E3 14.7-kilodalton protein with a novel tumor necrosis factor alpha-inducible cellular protein containing leucine zipper domains. Mol. Cell. Biol. **18**:1601–1610.
48. **Lillie, J. W., P. M. Loewenstein, M. R. Green, and M. Green.** 1987. Functional domains of adenovirus type 5 E1a proteins. Cell **50**:1091–1100.
49. **Mahr, J. A., and L. R. Gooding.** 1999. Immune evasion by adenoviruses. Immunol. Rev. **168**:121–130.
50. **Mariani, S. M., and P. H. Krammer.** 1998. Surface expression of TRAIL/Apo-2 ligand in activated mouse T and B cells. Eur. J. Immunol. **28**:1492–1498.
51. **Mariani, S. M., B. Matiba, E. A. Armandola, and P. H. Krammer.** 1997. Interleukin 1β-converting enzyme related proteases/caspases are involved in TRAIL-induced apoptosis of myeloma and leukemia cells. J. Cell Biol. **137**:221–229.
52. **Martinez-Lorenzo, M. J., M. A. Alava, S. Gamen, K. J. Kim, A. Chuntharapai, A. Pineiro, J. Naval, and A. Anel.** 1998. Involvement of APO2 ligand/TRAIL in activation-induced death of Jurkat and human peripheral blood T cells. Eur. J. Immunol. **28**:2714–2725.
53. **Nagata, S.** 1997. Apoptosis by death factor. Cell **88**:355–365.
54. **Pan, G., J. Ni, Y. F. Wei, G. Yu, R. Gentz, and V. M. Dixit.** 1997. An antagonist decoy receptor and a death domain-containing receptor for TRAIL. Science **277**:815–818.
55. **Pan, G., K. O'Rourke, A. M. Chinnaiyan, R. Gentz, R. Ebner, J. Ni, and V. M. Dixit.** 1997. The receptor for the cytotoxic ligand TRAIL. Science **276**:111–113.
56. **Perez, D., and E. White.** 1998. E1B 19K inhibits Fas-mediated apoptosis through FADD-dependent sequestration of FLICE. J. Cell Biol. **141**:1255–1266.
57. **Perez, D., and E. White.** 2000. TNFα signals apoptosis through a Bid-dependent conformational change in Bax that is inhibited by E1B 19K. Mol. Cell **6**:53–63.
58. **Pitti, R. M., S. A. Marsters, S. Ruppert, C. J. Donahue, A. Moore, and A. Ashkenazi.** 1996. Induction of apoptosis by Apo-2 ligand, a new member of the tumor necrosis factor cytokine family. J. Biol. Chem. **271**:12687–12690.
59. **Ranheim, T. S., J. Shisler, T. M. Horton, L. J. Wold, L. R. Gooding, and W. S. M. Wold.** 1993. Characterization of mutants within the gene for the adenovirus E3 14.7-kilodalton protein which prevents cytolysis by tumor necrosis factor. J. Virol. **67**:2159–2167.
60. **Routes, J. M., S. Ryan, A. Clase, T. Miura, A. Kuhl, T. A. Potter, and J. L. Cook.** 2000. Adenovirus E1A oncogene expression in tumor cells enhances killing by TNF-related apoptosis-inducing ligand. J. Immunol. **165**:4522–4527.

61. **Sabelko-Downes, K., and J. H. Russell.** 2000. The role of fas ligand in vivo as a cause and regulator of pathogenesis. Curr. Opin. Immunol. **12**:330–335.
62. **Screaton, G., and X.-N. Xu.** 2000. T cell life and death signalling via TNF-receptor family members. Curr. Opin. Immunol. **12**:316–322.
63. **Sedger, L., D. Shows, R. Blanton, J. Peschon, R. Goodwin, D. Cosman, and S. Wiley.** 1999. IFN-γ mediates a novel antiviral activity through dynamic modulation of TRAIL and TRAIL receptor expression. J. Immunol. **163**: 920–926.
64. **Sheridan, J. P., S. A. Marsters, R. M. Pitti, A. Gurney, M. Skubatch, D. Baldwin, L. Ramakrishnan, C. L. Gray, K. Baker, W. I. Wood, A. D. Goddard, and A. Ashkenazi.** 1997. Control of TRAIL-induced apoptosis by a family of signaling and decoy receptors. Science **277**:818–821.
65. **Shisler, J., C. Yang, B. Walter, C. F. Ware, and L. R. Gooding.** 1997. The adenovirus E3-10.4K/14.5K complex mediates loss of cell surface Fas (CD95) and resistance to Fas-induced apoptosis. J. Virol. **71**:8299–8306.
66. **Sparer, T., R. A. Tripp, D. L. Dillehay, T. W. Hermiston, W. S. M. Wold, and L. R. Gooding.** 1996. The role of adenovirus early region 3 proteins (gp19K, 10.4K, 14.5K, and 14.7K) in a murine pneumonia model. J. Virol. **70**:2431–2439.
67. **Stewart, A. R., A. E. Tollefson, P. Krajcsi, S. P. Yei, and W. S. M. Wold.** 1995. The adenovirus E3 10.4K and 14.5K proteins, which function to prevent cytolysis by tumor necrosis factor and to down-regulate the epidermal growth factor receptor, are localized in the plasma membrane. J. Virol. **69**:172–181.
68. **Subramanian, T., M. Kuppuswamy, J. Gysbers, S. Mak, and G. Chinnadurai.** 1984. 19-kDa tumor antigen coded by early region E1b of adenovirus 2 is required for efficient synthesis and for protection of viral DNA. J. Biol. Chem. **259**:11777–11783.
69. **Teodoro, J. G., and P. E. Branton.** 1997. Regulation of apoptosis by viral gene products. J. Virol. **71**:1739–1746.
70. **Thomas, W. D., and P. Hersey.** 1998. TNF-related apoptosis-inducing ligand (TRAIL) induces apoptosis in Fas ligand-resistant melanoma cells and mediates CD4 T cell killing of target cells. J. Immunol. **161**:2195–2200.
71. **Thorne, T. E., C. Voelkel-Johnson, W. M. Casey, L. W. Parks, and S. M. Laster.** 1996. The activity of cytosolic phospholipase A_2 is required for the lysis of adenovirus-infected cells by tumor necrosis factor. J. Virol. **70**:8502–8507.
72. **Tollefson, A. E., T. W. Hermiston, D. L. Lichtenstein, C. F. Colle, R. A. Tripp, T. Dimitrov, K. Toth, C. E. Wells, P. C. Doherty, and W. S. M. Wold.** 1998. Forced degradation of Fas inhibits apoptosis in adenovirus-infected cells. Nature **392**:726–730.
73. **Tollefson, A. E., T. W. Hermiston, and W. S. M. Wold.** 1998. Preparation and titration of CsCl-banded adenovirus stocks., p. 1–9. *In* W. S. M. Wold (ed.), Adenovirus methods and protocols. The Humana Press, Totowa, N.J.
74. **Tollefson, A. E., P. Krajcsi, M. H. Pursley, L. R. Gooding, and W. S. M. Wold.** 1990. A 14,500 MW protein is coded by region E3 of group C human adenoviruses. Virology **175**:19–29.
75. **Tollefson, A. E., P. Krajcsi, S. P. Yei, C. R. Carlin, and W. S. M. Wold.** 1990. A 10,400-molecular-weight membrane protein is coded by region E3 of adenovirus. J. Virol. **64**:794–801.
76. **Tollefson, A. E., A. Scaria, T. W. Hermiston, J. S. Ryerse, L. J. Wold, and W. S. M. Wold.** 1996. The adenovirus death protein (E3-11.6K) is required at very late stages of infection for efficient cell lysis and release of adenovirus from infected cells. J. Virol. **70**:2296–2306.
77. **Tollefson, A. E., A. R. Stewart, S. P. Yei, S. K. Saha, and W. S. M. Wold.** 1991. The 10,400- and 14,500-dalton proteins encoded by region E3 of adenovirus form a complex and function together to down-regulate the epidermal growth factor receptor. J. Virol. **65**:3095–3105.
78. **Tollefson, A. E., and W. S. M. Wold.** 1988. Identification and gene mapping of a 14,700-molecular-weight protein encoded by region E3 of group C adenoviruses. J. Virol. **62**:33–39.
79. **van Weert, A. W. M., K. W. Dunn, H. J. Geuze, F. R. Maxfield, and W. Stoorvogel.** 1995. Transport from late endosomes to lysosomes, but not sorting of integral membrane proteins in endosomes, depends on the vacuolar proton pump. J. Cell Biol. **130**:821–834.
80. **Vidalain, P., O. Azocar, B. Lamouille, A. Astier, C. Rabourdin-Combe, and C. Servet-Delprat.** 2000. Measles virus induces functional TRAIL production by human dendritic cells. J. Virol. **74**:556–559.
81. **Walczak, H., M. A. Degli-Esposti, R. S. Johnson, P. J. Smolak, J. Y. Waugh, N. Boiani, M. S. Timour, M. J. Gerhart, K. A. Schooley, C. A. Smith, R. G. Goodwin, and C. T. Rauch.** 1997. TRAIL-R2: a novel apoptosis-mediating receptor for TRAIL. EMBO J. **16**:5386–5397.
82. **Walczak, H., and P. H. Krammer.** 2000. The CD95 (APO-1/Fas) and the TRAIL (APO-2L) apoptosis system. Exp. Cell Res. **256**:58–66.
83. **White, E.** 1998. Regulation of apoptosis by adenovirus E1A and E1B oncogenes. Semin. Virol. **8**:505–513.
84. **White, E., P. Sabbatini, M. Debbas, W. S. M. Wold, D. I. Kusher, and L. R. Gooding.** 1992. The 19-kilodalton adenovirus E1B transforming protein inhibits programmed cell death and prevents cytolysis by tumor necrosis factor α. Mol. Cell. Biol. **12**:2570–2580.
85. **Wiley, S. R., K. Schooley, P. J. Smolak, W. S. Din, C.-P. Huang, J. K. Nicholl, G. R. Sutherland, T. D. Smith, C. Rauch, C. A. Smith, and R. G. Goodwin.** 1995. Identification and characterization of a new member of the TNF family that induces apoptosis. Immunity **3**:673–682.
86. **Wissing, D., H. Mouritzen, M. Egeblad, G. G. Poirier, and M. Jaattela.** 1997. Involvement of caspase-dependent activation of cytosolic phospholipase A_2 in tumor necrosis factor-induced apoptosis. Proc. Natl. Acad. Sci. USA **94**:5073–5077.
87. **Wold, W. S. M., and G. Chinnadurai.** 2000. Adenovirus proteins that regulate apoptosis, p. 200–232. *In* A. J. Cann (ed.), DNA virus replication: frontiers in molecular biology. Oxford University Press, Oxford, United Kingdom.
88. **Wold, W. S. M., S. L. Deutscher, N. Takemori, B. M. Bhat, and S. C. Magie.** 1986. Evidence that AGU*AUAUG*A and CC*AAGAUG*A initiate translation in the same mRNA region E3 of adenovirus. Virology **148**:168–180.
89. **Wold, W. S. M., K. Doronin, K. Toth, M. Kuppuswamy, D. L. Lichtenstein, and A. E. Tollefson.** 1999. Immune responses to adenoviruses: viral evasion mechanisms and their implications for the clinic. Curr. Opin. Immunol. **11**:380–386.
90. **Wold, W. S. M., and A. E. Tollefson.** 1998. Adenovirus E3 proteins: 14.7K, RID, and gp19K inhibit immune-induced cell death; adenovirus death protein promotes cell death. Semin. Virol. **8**:515–523.
91. **Yoshimori, T., A. Yamamoto, Y. Moriyama, M. Futai, and Y. Tashiro.** 1991. Bafilomycin A1, a specific inhibitor of vacuolar-type H+-ATPase, inhibits acidification and protein degradation in lysosomes of cultured cells. J. Biol. Chem. **266**:17707–17712.
92. **Zilli, D., C. Voelkel-Johnson, T. Skinner, and S. M. Laster.** 1992. The adenovirus E3 region 14.7 kDa protein, heat and sodium arsenite inhibit the TNF-induced release of arachidonic acid. Biochem. Biophys. Res. Commun. **188**:177–183.